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1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
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Absorption, Distribution, Metabolism, and Excretion of 14C-RDX Following Oral Administration to Minipigs

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6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Covance Laboratories Inc.
3301 Kinsman Boulevard
Madison, WI 53704-2595

8. PERFORMING ORGANIZATION REPORT NUMBER

Covance 7273-121

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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Health Effects Research Program
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10. SPONSOR/MONITOR'S ACRONYM(S)

Gunda Reddy, PhD, DABT

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The tissue distribution, elimination, and metabolism of radioactivity were examined following a single oral administration of 14C-RDX, at a target dose of 45 mg/kg, to male and female Yucatan minipigs. Blood, urine, and feces were collected through 24 hours postdose. Blood, plasma, selected tissues, urine, and feces were analyzed for total radioactivity. Following dose administration, all animals vomited. Animals vomited at various times over the course of the study. Concentrations of 14C-RDX-derived radioactivity in plasma reached maximum levels (Cmax) at 12 hours postdose for both males and one female. Blood and plasma concentrations for the second female reached Cmax at 6 hours postdose. The plasma Cmax values for males ranged from 8.76 to 18.3 ug equivalents 14C-RDX/g, and in females values ranged from 8.54 to 10.0 ug equivalents 14C-RDX/g. The distribution of 14C-RDX-derived radioactivity was extensive, with drug derived radioactivity quantifiable in all analyzed tissues at 24 hours postdose. (continued)

15. SUBJECT TERMS

Yucatan mini pigs, 14-C-RDX, Cyclotrimetylenetrinitramine,Toxicokinetics, Metabolism, 4-nitro-2-4-diazabutanal, 4-nitro-2,4-diaza-butanamide

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Dr. Gunda Reddy
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14. ABSTRACT (continued)

The highest concentrations of radioactivity were observed in liver, kidneys, and small intestine. The lowest values were observed in abdominal fat, skeletal muscle, and skin. Quantifiable levels of radioactivity in the brain and testes suggest 14C RDX derived radioactivity crosses the blood/brain and blood/testes barriers. Urine was the major route of elimination of 14C-RDX-derived radioactivity. At 24 hours postdose, urine and feces accounted for average values of 17.3 and 0.53% of the dosed radioactivity, respectively, in males and in females accounted for average values of 16.0 and 0.79% of the dosed radioactivity, respectively. The overall excretion recoveries in males and females were 40.6 and 29.7%, respectively. The relatively low levels of radioactivity observed in gastrointestinal contents and wash as well as low levels in feces suggest nearly complete oral absorption of 14C-RDX-derived radioactivity. Metabolite profiling and LC/MS/MS showed quantifiable levels of metabolites M1 (4-nitro-2,4-diazabutanal), M2 (4-nitro-2,4-diaza-butanamide), and parent RDX. All three metabolites were observed in urine, only RDX was observed in plasma. LC/MS/MS analysis of plasma showed trace amounts of RDX metabolites MNX, DNX, and TNX. Analysis also showed trace amounts of the metabolites MNX and DNX in male urine and MNX in female urine. RDX concentrations in the brains ranged from 33.5 to 1070 ng/g. RDX concentrations in liver were less than 1 ng/g.

Final Report

Study Title	Absorption, Distribution, Metabolism, and Excretion of ^{14}C-RDX Following Oral Administration to Minipigs
Data Requirement	40 CFR 160, Guideline OPPTS 870.7485
Authors	Timothy J. Musick, PhD Milan A. Berge, PhD Shari S. Patzer Karen R. Tilch
Sponsor	U.S. Army Center for Health Promotion and Preventive Medicine Building E2100 Aberdeen Proving Ground, MD 21010
Sponsor Study Coordinator	Gunda Reddy, PhD, DABT
Contract Number	P-2319
Study Location	Covance Laboratories Inc. 3301 Kinsman Boulevard Madison, WI 53704-2595
Covance Study Identification	Covance 7273-121 CMS 29676E
Report Issued	Final, 02 August 2010
Page Number	1 of 145

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Absorption, Distribution, Metabolism, and Excretion of ^{14}C-RDX Following Oral Administration to Minipigs

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d)(1) (A), (B), or (C).

Company: U.S. Army Center for Health Promotion and Preventive Medicine

GUNDA REDDY
Company Agent:

TOXICOLOGIST
Title:

Signature

July 30, 2010
Date

These data are the property of U.S. Army Center for Health Promotion and Preventive Medicine and, as such, are considered to be confidential for all purposes other than compliance with FIFRA §10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality that may exist under any other statute or in any other country.

COMPLIANCE STATEMENT

Absorption, Distribution, Metabolism, and Excretion of ^{14}C-RDX Following Oral Administration to Minipigs

All aspects of this study were in accordance with the Environmental Protection Agency Good Laboratory Practice Standards (40 CFR 160) and the Wisconsin Department of Health and Family Services, Radiation Protection Section (License No. 025-1076-01) with the following exceptions.

- The protocol was signed on 13 July 2004. During the time between 06 July 2004 and 13 July 2004, prestudy activities, including general husbandry were conducted.
- The diet fed to the pigs was not a certified diet; therefore, it was not analyzed for environmental contaminants.
- The paper data for an analysis run on 11 October 2004, which included the injection record and chromatograms, could not be located. The paper data was reconstructed by reprinting the electronic files. The reference substance data from this analysis were only used to demonstrate retention characteristics. No other data from this analysis run were reported.
- The reference substances used in this study were not synthesized or characterized in accordance with GLP regulations. No lot numbers were assigned and the stability was not determined.
- The TNX, MNX, DNX, and MEDINA reference substances were received at Covance during preliminary study discussions. The exact date of receipt storage of these materials at Covance and the storage of the materials prior to 29 January 2003 were not documented and cannot be verified.
- The amount of sodium acetate buffer used for the β-glucuronidase solution cannot be verified; however, the positive control for this assay indicates that the enzyme was active.
- The cold concentration analysis portion of the study were conducted in accordance with generally recognized good laboratory practices, but were not considered to be within the scope of the Good Laboratory Practice regulations.

In the opinion of the Study Director, these deviations do not negatively impact the interpretation of the data or the outcome of the study.

Timothy J. Musick, PhD
Study Director
Drug Metabolism
Covance Laboratories Inc.

02 August 2010
Date

Frederic W. Thalacker, PhD
Director
Drug Metabolism
Covance Laboratories Inc.

02 August 2010
Date

Gunda Reddy, PhD, DABT
U.S. Army Center for Health Promotion and
Preventive Medicine

July 30, 2010
Date

QUALITY ASSURANCE STATEMENT

This report has been reviewed by the Quality Assurance Unit of Covance Laboratories Inc. and accurately reflects the raw data. The following study specific inspections were conducted and findings reported to the study director (SD) and associated management.

Inspection Dates		Phase	Date Reported to SD and SD Management
From	To		
14 Jul 2004	15 Jul 2004	Protocol Review	15 Jul 2004
12 Aug 2004	12 Aug 2004	Protocol Amendment Review	12 Aug 2004
16 Aug 2004	16 Aug 2004	Test Article Administration/Application	16 Aug 2004
12 Jan 2005	12 Jan 2005	Metabolite Profiling	12 Jan 2005
27 May 2005	16 Jun 2005	Data Review	17 Jun 2005
27 May 2005	16 Jun 2005	Draft Report Review	17 Jun 2005
28 Nov 2005	08 Dec 2005	Data Review	08 Dec 2005
28 Nov 2005	08 Dec 2005	Revised Draft Report Review	08 Dec 2005
23 Nov 2009	08 Dec 2009	Revised Draft Report and Data Review	08 Dec 2009
19 May 2010	19 May 2010	Protocol Amendment Review	19 May 2010
30 Jul 2010	30 Jul 2010	Revised Draft Report Review	30 Jul 2010

J Paul Mitchell — 02 August 2010

Representative
Quality Assurance Unit

Date

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ABSTRACT

The tissue distribution, elimination, and metabolism of radioactivity were examined following administration of a single oral dose of ^{14}C-RDX at a target dose of 45 mg/kg to two male and two female Yucatan minipigs. Blood was collected at 1, 6, 12, and 24 hours postdose, and plasma was prepared by centrifugation. Urine was collected predose and at 0-6, 6-12, and 12-24 hours postdose. Feces were collected predose and at 0-24 hours postdose. Tissues were collected at 24 hours postdose. Blood, plasma, selected tissues, urine, and feces were analyzed for total radioactivity. Selected plasma, urine, and liver samples were also analyzed for metabolite profiles.

Concentrations of ^{14}C-RDX-derived radioactivity in blood and plasma reached maximum levels (C_{max}) at 12 hour postdose for both males and one female. Blood and plasma concentrations for the second female reached C_{max} at 6 hours postdose. In males, C_{max} values in blood ranged from 7.18 to 14.4 μg equivalents ^{14}C-RDX/g. In females, these values ranged from 6.49 to 7.90 μg equivalents ^{14}C-RDX/g. The plasma C_{max} values for males ranged from 8.76 to 18.3 μg equivalents ^{14}C-RDX/g, and in females values ranged from 8.54 to 10.0 μg equivalents ^{14}C-RDX/g. All animals vomited at various times and in varying amounts during the study. The data must therefore, be viewed with this in mind as this will have an effect on the variability in concentrations between animals.

The distribution of ^{14}C-RDX-derived radioactivity was extensive, with drug derived radioactivity quantifiable in all analyzed tissues at 24 hours postdose. The highest concentrations of ^{14}C-RDX-derived radioactivity in both males and females were observed in liver, kidneys, and small intestine for all animals. The lowest values were observed in abdominal fat, skeletal muscle, and skin, with average values no higher than 5.67 μg equivalents ^{14}C-RDX/g. Quantifiable levels of radioactivity in the brain and testes suggests ^{14}C-RDX-derived radioactivity crosses the blood/brain and blood/testes barriers.

For all animals, the tissue:plasma concentration ratios were greater than one for kidneys, liver, and small intestine, and less than one for blood, brain, abdominal fat, heart, skeletal muscle, and skin.

The highest percentages of radioactive dose in tissues were observed in liver, skeletal muscle, stomach contents and wash, and abdominal fat, with average values of 2.79 to 5.80% of the administered dose in males. In females, the highest percentages of radioactive dose were found in liver and skeletal muscle, with values of 3.48 and 2.47% of the administered dose, respectively.

Urine was the major route of elimination of ^{14}C-RDX-derived radioactivity in both males and females. At 24 hours postdose, in males, urine and feces accounted for average values of 17.3 and 0.53% of the dosed radioactivity, respectively, and in females accounted for average values of 16.0 and 0.79% of the dosed radioactivity, respectively. The overall excretion recoveries in males and females were 40.6 and 29.7%, respectively.

The relatively low levels of radioactivity observed in gastrointestinal contents and wash as well as low levels in feces suggest nearly complete oral absorption of ^{14}C-RDX-derived radioactivity.

Metabolite profiling and identification by LC/MS showed quantifiable levels of metabolites M1 (4-nitro-2,4-diazabutanal), M2 (4-nitro-2,4-diaza-butanamide), and parent RDX. All three were observed in the urine samples, with only RDX observed in plasma. None of the liver extract radioactivity showed quantifiable levels of RDX or identified metabolites. Most of the profiled radioactivity was not identified and was observed at or near the void volume in methods using reverse phase columns and a mixed phase column utilizing reverse phase and size exclusion. This suggests that most of the radioactivity is very water-soluble and of higher molecular weight.

LC/MS/MS analysis of plasma showed trace amounts of RDX metabolites MNX, DNX, and TNX; however, no quantifiable levels of radioactivity were observed. Analysis also showed trace amounts of the RDX metabolites MNX and DNX in male urine and MNX in female urine; however, no quantifiable levels of radioactivity were observed.

Quantitative analysis of RDX in liver and brain at 24 hours postdose was performed by LC/MS/MS. Concentrations of RDX in the brains ranged from 33.5 to 200 ng/g in females and 419 to 1070 ng/g in males. RDX concentrations in liver were less than 1 ng/g.

TITLE

Absorption, Distribution, Metabolism, and Excretion of ^{14}C-RDX Following Oral Administration to Minipigs

OBJECTIVE

The purpose of this study was to assess the extent of absorption, distribution, metabolism, and excretion of radioactivity following administration of a single dose of ^{14}C-RDX given to pigs by an oral route of administration. Selected samples were analyzed for metabolite profiles.

REGULATORY COMPLIANCE

This study was conducted by Covance Laboratories Inc. (Covance), 3301 Kinsman Boulevard, Madison, Wisconsin, in accordance with Covance Protocol CMS 29676E dated 13 July 2004, Protocol Amendment No. 1, and Covance standard operating procedures (SOPs). The protocol, amendment, and protocol deviations are in Appendix 1. The study was conducted in accordance the Environmental Protection Agency (EPA) Good Laboratory Practice Standards (40 CFR 160), and in compliance with the following testing guidelines: United States Environmental Protection Agency (EPA) FIFRA, 40 CFR 158, Health Effects Test Guidelines (OPPTS 870.7485); the Wisconsin Department of Health and Family Services, Radiation Protection Section (License No. 025-1076-01); with the following exceptions.

- The protocol was signed on 13 July 2004. During the time between 06 July 2004 and 13 July 2004, prestudy activities, including general husbandry were conducted.
- The diet fed to the pigs was not a certified diet; therefore, it was not analyzed for environmental contaminants.
- The paper data for an analysis run on 11 October 2004, including the injection record and chromatograms could not be located. The paper data was reconstructed by reprinting the electronic files. The reference substance data from this analysis were only used to demonstrate retention characteristics. No other data from this analysis run were reported.
- The reference substances used in this study were not synthesized or characterized in accordance with GLP regulations. No lot numbers were assigned and the stability was not determined.
- The TNX, MNX, DNX, and MEDINA reference substances were received at Covance during preliminary study discussions. The exact date of receipt storage of these materials at Covance and the storage of the materials prior to 29 January 2003 were not documented and cannot be verified.

- The amount of sodium acetate buffer used for the β-glucuronidase solution cannot be verified; however, the positive control for this assay indicates that the enzyme was active.

- The cold concentration analysis portion of the study were conducted in accordance with generally recognized good laboratory practices, but were not considered to be within the scope of the Good Laboratory Practice regulations.

All procedures in the study were in compliance with the Animal Welfare Act Regulations (9 CFR 3).

The protocol, study conduct, data, and final report were audited by the Quality Assurance Unit (QAU) of Covance in accordance with Covance SOPs.

The major computer systems used for this study included the following. The software version numbers are recorded in the raw data.

System	Function
Path Tox (PTS)	Direct on-line capture of in-life toxicology data
Randomization and Data Extension System (RADES)	Used in conjunction with PTS to randomize animals for assignment to treatment groups
Debra	An automated and validated data capture and management system for data collection from balances and scintillation counters for studies using radiolabeled test articles
Metasys	Monitors environmental conditions in the animal facility
Rees	Monitors environmental conditions in storage units
HP Chemstation and Radiomatic Flo-One	Used to capture data from liquid chromatography systems
MassLynx and Analyst	Capture of liquid chromatography/mass spectrometry data

TEST ARTICLES AND REFERENCE SUBSTANCES

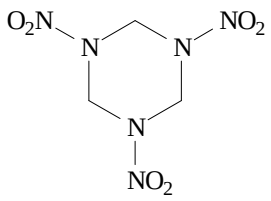
The radiolabeled and nonradiolabeled test articles were provided by the Sponsor and received at Covance on 22 July 2004. Information specific to the test articles follows.

Radiolabeled Test Article

Test article:	^{14}C-RDX (RDX, [^{14}C(U)]-)
Chemical name:	Cyclotrimethylenetrinitramine
Lot No.:	3501-065
Specific activity:	39.20 mCi/mmol
Conditions:	Wetted with 5 µL of water on day of receipt.
Radiopurity:	99.37%
Storage conditions:	In a freezer set to maintain –10 to –30°C

Nonradiolabeled Test Article

Test article:	RDX	Structure:
Chemical name:	Cyclotrimethylenetrinitramine	
CAS number:	121-82-4	
Molecular formula:	$C_3H_6N_6O_6$	
Molecular weight:	222.1	
Lot No.:	0858S00	
Chemical purity:	>99.5%	
Conditions:	Received wetted with 5% water	
Storage conditions:	Ambient temperature	



Reference Substances

In addition to the radiolabeled and nonradiolabeled test articles, the following references substances were used. The TNX, MNX, DNX, MEDINA, and 4-nitro-2,4-diazabutanal reference substances were received from SRI International. Information specific to the references substances follows.

Reference substance:	1,3,5,-trinitroso-1,3,5-triazacyclohexane (TNX)
Purity as received:	>99.9%
Storage conditions:	In a freezer set to maintain -10 to -30°C, protected from light (see protocol deviations)
Reference substance:	1-nitroso-3,5-dinitro-1,3,5-triazacyclohexane (MNX)
Purity as received:	>98.4% (contains approximately 1.2% RDX)
Storage conditions:	In a freezer set to maintain -60 to -90°C (see protocol deviations)
Reference substance:	1-nitro-3,5-dinitroso-1,3,5-triazacyclohexane (DNX)
Purity as received:	>67% (contains approximately 24% MNX and 7% TNX)
Storage conditions:	In a freezer set to maintain -60 to -90°C (see protocol deviations)
Reference substance:	Methylenedinitramine (MEDINA)
Purity as received:	>99%
Storage conditions:	In a freezer set to maintain -10 to -30°C (see protocol deviations)
Reference substance:	4-nitro-2,4-diazabutanal
Purity as received:	>99%
Storage conditions:	In a freezer set to maintain -10 to -30°C

Determination of Radiopurity

Prior to dose preparation, the radiochemical purity of the test article was verified at Covance by high performance liquid chromatography (HPLC). The method is in Appendix 2.

Reserve Samples
Since the experimental portion of the study was more than four weeks in duration, the Sponsor assumed responsibility for retention of samples of the test articles and reference substances in accordance with the applicable regulations.

EXPERIMENTAL DESIGN AND PROCEDURES

The in-life portion of the study was conducted 16 August through 17 August 2004. The experimental start date was 16 August 2004 and the experimental end date was 13 May 2005.

Study Design
Four pigs were assigned to this study. At designated time points following dosing, blood, urine, feces, and selected tissues were collected. The number of animals, target dose level, and target dose volume were as follows:

	Number of Animals		Target Dose Level	Dose	Target Dose Volume	Samples
Group	Male	Female	(mg/kg)	Route	(mL/kg)	Collected
1	2	2	45	Oral	5	Blood, tissues, urine, and feces

Note: Each animal received a target dose of approximately 50-75 μCi/kg.

Reason for Dosing Route
Potential exposure is by the oral route.

Test Animals and Housing
Two male and two female Yucatan minipigs, from Sinclair Research Center, Inc., Columbia, MO, were received on 06 July 2004. The animals were acclimated for approximately 6 weeks prior to dose administration. At dosing, the animals weighed 14.7 to 16.4 kg and were approximately 4 months of age.

All animals were housed in individual, stainless steel cages during acclimation. During the test period, animals were housed in individual stainless steel metabolism cages designed for the separation and collection of urine and feces.

Approximately 500 g of #5082 mini pig diet (PMI) was provided twice daily through 20 July 2004. In an effort to slow the weight gain of the pigs, necessary because of a delay in dosing, starting 21 July 2004, animals were provided approximately 300 g of food twice daily through the remainder of the study. This diet was not a certified diet; therefore, it was not analyzed for environmental contaminants. Appropriate treats (that do not require analysis) were offered in accordance with Covance SOPs for environmental/psychological enrichment. Water was provided fresh daily *ad libitum*.

Identification and Randomization
Upon arrival, each animal was assigned a unique identification number. Each pig was identified with an implantable microchip device.

Dose Preparation, Verification, and Stability
On the day of receipt, 5 µL of water was added to ^{14}C-RDX. On the day of dosing, 30.086 mg of ^{14}C-RDX and 4,218.6 mg of RDX were combined with 450 mL of 0.5% carboxymethylcellulose in water. The dose formulation was stirred and sonicated until a homogeneous suspension was obtained.

Duplicate weighed aliquots were taken from the dose formulation prior to and following dose administration, and were analyzed by liquid scintillation counting (LSC) to determine the concentration of radioactivity and homogeneity.

Stability of the bulk test article was the responsibility of the Sponsor. Stability under conditions of administration was demonstrated by analyzing predose and postdose aliquots by HPLC.

Dose Administration
The volume of radiolabeled dose formulation to be administered to each animal was calculated based on the body weight taken on the day of dosing. The actual amount administered was determined by weighing the dose syringe before and after dose administration. The residual radioactivity recovered from the dose apparatus was subtracted from the dispensed amount to determine the actual dose administered.

Animals were not fasted prior to dosing. The oral dose was administered via oral gavage. Prior to withdrawing the gavage tube, the tube was flushed with approximately 5 mL of dose vehicle. Each gavage tube was retained for radioanalysis.

Observation of Animals
Antemortem Observations
Twice daily (a.m. and p.m.), animals were observed for mortality and signs of pain and distress. Cageside observations for general health and appearance were done once daily.

Body Weights
Animals were weighed at arrival, weekly during acclimation, at randomization, and on the day of dose administration.

Sample Collection
The following samples were collected for radioanalysis.

Excretion
The following samples were collected for analysis of excretion of radioactivity.

Urine was collected from all animals predose (at least 12 hours) and at 0-6, 6-12, and 12-24 hours postdose. Urine was collected in plastic containers surrounded by dry ice. The weight of each sample was recorded using the Debra system.

Feces were collected predose (at least 12 hours) and at 0-24 hours postdose. Feces were collected at room temperature and transferred to plastic containers and stored in a freezer

set to maintain -10 to -30°C. The weight of each sample was recorded using the Debra system.

Vomitus was wiped from the cages using gauze pads. In addition, for Animals M01640, M01643, and M01644, the cage area where vomitus occurred was rinsed with water, and the water collected into the vomitus container. The vomitus samples were pooled, as appropriate, and submitted for radioanalysis.

After the last excreta collections, cages were washed and wiped with a solution of 1% trisodium phosphate in water and gauze pads. The cage wash samples and gauze were collected into separate plastic containers and the weight of each cage wash sample was recorded using the Debra system.

Blood and Plasma

Blood (approximately 10 mL) was collected via the anterior vena cava into tubes containing sodium heparin anticoagulant from all animals at 1, 6, and 12 hours postdose. Blood samples were placed on wet ice, in a chilled Kryorack, or stored at in a refrigerator set to maintain 3 to 5°C until aliquoted and centrifuged to obtain plasma, buffy coat, and cellular fraction.

Tissue Distribution

At 24 hours postdose, animals were sacrificed via exsanguination under sodium pentobarbital anesthesia (see protocol deviations). Animals were anesthetized with telazol prior to sacrifice and after blood collections. Prior to sacrifice, blood (approximately 30 mL) was collected via the anterior vena cava into tubes containing sodium heparin anticoagulant. Blood samples were placed on wet ice, in a chilled Kryorack, or stored in a refrigerator set to maintain 3 to 5°C until aliquoted and centrifuged to obtain plasma, buffy coat, and cellular fraction.

The following matrices were collected from each animal, as appropriate:

Brain	Liver	Small intestine contents and wash
Fat (abdominal)	Lungs	Stomach
Heart	Muscle (skeletal)	Stomach contents and wash
Kidneys	Ovaries (as applicable)	Testes (as applicable)
Large intestine	Skin	
Large intestine contents and wash	Small intestine	

Tissues were excised, rinsed with saline and blotted dry (as appropriate), weighed, and placed on dry ice. In addition, 5 approximately 2-3 g samples of each liver were flash frozen in liquid nitrogen for possible shipment to the Sponsor. The residual carcass was discarded.

Sample Identification, Storage, and Preparation

Sample Identification

Samples were identified with the Covance study number, sex, radioisotope, animal number, sample matrix, sample number, and collection time or interval.

Sample Storage
All samples, except blood, were stored in a freezer set to maintain -10 to -30°C before and after analysis. Blood was stored on wet ice, in a chilled Kryorack, or in a refrigerator set to maintain 3 to 5°C until aliquots were taken for radioanalysis. Plasma was harvested by centrifugation and stored in a freezer set to maintain -10 to -30°C. The cellular fraction of the blood and the buffy coat were stored in a freezer set to maintain -10 to -30°C, but were not analyzed.

Sample Preparation and Radioanalysis
All sample combustions were done in a Model 307 Sample Oxidizer (Packard Instrument Company) and the resulting $^{14}CO_2$ was trapped in a mixture of Perma Fluor and Carbo-Sorb. Ultima Gold XR scintillation cocktail was used for samples analyzed directly. All samples were analyzed for radioactivity in Model 2900TR liquid scintillation counters (Packard Instrument Company) for at least 5 minutes or 100,000 counts. Each sample was homogenized before radioanalysis (unless the entire sample was used for analysis). All samples were analyzed in duplicate if sample size allowed. If results from sample duplicates (calculated as ^{14}C dpm/g sample) differed by more than 10% from the mean value, the sample was rehomogenized and reanalyzed (if the sample size permitted). This specification was met for all sample aliquots that had radioactivity greater than 100 dpm.

Scintillation counting data (cpm) were automatically corrected for counting efficiency using the external standardization technique and an instrument-stored quench curve generated from a series of sealed quenched standards.

Blood and Plasma
Blood was mixed by inverting several times and duplicate weighed aliquots were combusted and analyzed by LSC. The remaining blood was centrifuged at approximately 2400 rpm (1300 x g) for approximately 10 minutes at approximately 5°C.

The buffy coat was transferred to prelabeled vials. The plasma was mixed and duplicate weighed aliquots were analyzed directly by LSC. The cellular fraction was transferred to prelabeled vials.

Brain, Heart, Kidneys, Liver, Lungs, Ovaries, and Testes
Each sample was cut into small pieces, frozen with liquid nitrogen, and homogenized in a food grinder. Duplicate weighed aliquots were combusted and analyzed by LSC.

Skin
A small amount of sample was saved for possible future analysis. The remaining sample was digested in sodium hydroxide until dissolved. Duplicate weighed aliquots were analyzed directly by LSC.

Fat (abdominal)
Samples were cut into small pieces, frozen in liquid nitrogen, and homogenized in a food grinder. Duplicate weighed aliquots were allowed to extract in scintillation cocktail at room temperature and analyzed by LSC.

Large Intestine, Muscle (skeletal), Small Intestine, and Stomach
Each sample was cut into pieces, which were frozen in liquid nitrogen and homogenized in a food grinder. A subsample was then digested in 1 N sodium hydroxide. Duplicate weighed aliquots were taken and 30% hydrogen peroxide was added to remove the color. After any foaming had dissipated, Ultima Gold XR scintillation cocktail was added, the aliquots were allowed to sit overnight and analyzed by LSC.

Large Intestine Contents, Small Intestine Contents, and Stomach Contents
An appropriate amount of acetonitrile was added, samples were shaken and allowed to extract. Each sample was homogenized with a probe-type homogenizer and duplicate weighed aliquots were removed while the sample was mixing; these aliquots were analyzed by LSC.

Urine
Samples were mixed by shaking and duplicate weighed aliquots were analyzed directly by LSC.

Feces
A sufficient amount of acetonitrile:water (50:50, v:v) was added to facilitate homogenization. Samples were homogenized with a probe-type homogenizer and duplicate weighed aliquots were combusted and analyzed by LSC.

Cage Wash
Samples were mixed and duplicate weighed aliquots were analyzed directly by LSC.

Cage Wipe, Dose Apparatus, and Vomitus
A sufficient amount of acetonitrile was added to cover each sample. Samples were allowed to extract, mixed by gentle shaking, and duplicate weighed aliquots were analyzed directly by LSC.

Metabolite Profiling
Urine Procedure
Urine samples were pooled by individual animal by combining aliquots from the 0-6, 6-12, and 12-24 hour collections based on a percentage (approximately 5% by weight) of the total urine collected for each animal at each time point. The pooled samples were weighed, mixed on a vortex mixer, and duplicate weighed aliquots were analyzed by LSC to determine radioactivity concentrations. A 1-mL aliquot of each pooled sample was transferred to a microfuge tube and centrifuged at approximately 20,000 relative centrifugal field (rcf). The resulting supernatant was transferred to a new vial and duplicate weighed aliquots were analyzed by LSC to determine the recovery of radioactivity. Radioactivity recoveries ranged from 95.1 to 97.5%. A portion of each supernatant was analyzed by HPLC using both Methods A and B.

Hydrolysis of Urine (ß-Glucuronidase/Sulfatase)
Pooled urine (0-24 hours) from male Animal M01638 was subjected to treatment with ß-glucuronidase (451,000-units/g glucuronidase and 13,500-units/g sulfatase activity). Two 2-mL aliquots of urine were placed into tubes and 1 mL of 0.4 M sodium acetate

buffer was added to each tube. The samples were mixed on a vortex mixer and then placed in a waterbath for approximately 15 minutes at approximately 37°C. To one tube, 1 mL of prewarmed 0.4 M sodium acetate buffer was added and to the other tube, 1 mL of ß-glucuronidase solution in 0.4 M sodium acetate buffer, pH 5.5 (35,000 units/mL), was added. The samples were incubated at approximately 37°C for 60 minutes. Acetonitrile (15 mL) was added to each sample. The samples were mixed on a vortex mixer and placed on ice for approximately 15 minutes. The samples were then centrifuged at approximately 3000 rcf for approximately 10 minutes at ambient temperature. Each supernatant was transferred to a new tube, and dried overnight at ambient temperature under a stream of nitrogen. Each sample was reconstituted in 236.7 mM sodium phosphate (pH 3). The remaining pellet from the centrifugation was rinsed with 236.7 mM sodium phosphate (pH 3) and the rinse combined with the reconstituted sample. The samples were then mixed on a vortex mixer and sonicated. The samples were transferred to a centrifuge tube and centrifuged at approximately 16,000 rcf for approximately 5 minutes. The supernatants were transferred to new vials and duplicate aliquots were analyzed by LSC to determine radioactivity concentration. Aliquots were also analyzed by HPLC using Method B.

Plasma Extraction (Method A)

Individual plasma samples (1, 6, 12, and 24 hours postdose) from male Animal M01638 and female Animal M01644 were prepared for analysis. Duplicate weighed aliquots of each plasma sample were analyzed by LSC to determine radioactivity concentrations. To an aliquot of each sample, methanol was added in a ratio of 1:3 (sample:methanol, v:v), and the sample mixed on a vortex mixer. The sample was then centrifuged at approximately 20,817 rcf for approximately 10 minutes at approximately 5°C. The resulting supernatant was transferred to a new vial. The extraction was repeated using the sample procedure, and the supernatants combined. The combined supernatant was weighed, and duplicate weighed aliquots were taken to determine extraction recovery. Extraction recoveries ranged from 96.4 to 39.4%. Each supernatant was concentrated under nitrogen at ambient temperature to approximately 0.5 mL. The concentrated samples were then filtered through a 0.45 µm Millipore filter unit using centrifugation at approximately 20,817 rcf for approximately 10 minutes at approximately 5°C. The filtrates were transferred to new vials and duplicate aliquots were analyzed by LSC to determine the concentration of radioactivity. A portion of each filtrate was analyzed by HPLC using Method A.

Plasma Extraction (Method B)

Individual plasma samples (1, 6, 12, and 24 hours postdose) from male Animal M01638 and female Animal M01644 were prepared for analysis. Duplicate aliquots were taken and analyzed by LSC to determine radioactivity concentration. An aliquot (approximately 0.5 mL) of each sample was filtered through a 0.45 µm Millipore filter unit using centrifugation at approximately 20,817 rcf for approximately 10 minutes at approximately 5°C. The filtrate was transferred to a new vial, and duplicate aliquots were analyzed by LSC to determine the recovery of radioactivity. Recoveries ranged from 103 to 60.8%, with the exception of the 24-hour female sample for which the recovery was 47.9%. A portion of each filtrate was analyzed by HPLC using Method B.

Liver Extraction

The liver samples from each animal were analyzed for metabolite profiles. To a 5-g aliquot of each liver, 20 mL of methanol was added and the sample mixed on a vortex mixer. The combined sample was sonicated, mixed on a vortex mixer, and then centrifuged at approximately 3000 rcf for approximately 10 minutes. The resulting supernatant was transferred to a new vial. The extraction was repeated using the sample procedure, and the supernatants combined. Duplicate weighed aliquots were taken and analyzed by LSC to determine extraction recovery. The extraction procedure was again repeated using 10 mL reverse osmosis water. Duplicate weighed aliquots were taken of each water supernatant and analyzed by LSC to determine extraction recovery. Each water supernatant was then combined with the respective methanol supernatant. The combined supernatants were dried overnight under a gentle stream of nitrogen. Hexane (5 mL) was added to each sample, and the sample mixed on a vortex mixer and sonicated for approximately 10 minutes. The samples were centrifuged at approximately 2000 rcf for 10 minutes, and then transferred to a freezer (approximately -70°C) for approximately 20 minutes. The hexane layer was removed and analyzed by LSC to determine radioactivity concentration. The remaining aqueous layer was evaporated to dryness under a gentle stream of nitrogen and then reconstituted with 2 mL methanol:water (1:1, v:v). The weight of the sample was recorded, and duplicate aliquots were taken and analyzed by LSC to determine radioactivity concentration. A weighed 500 µL aliquot was transferred to a 0.45 µm Millipore Ultrafree-MC filter and centrifuged at approximately 10,000 rcf. The resulting supernatant was transferred to a new vial and duplicate aliquots were taken and analyzed by LSC. The overall extraction recoveries ranged from 42.0 to 55.2%. All supernatants were analyzed using HPLC Method B. Supernatants from male Animal M01640 and female animal M01644 were also analyzed using HPLC Method A.

HPLC Procedure

Aliquots of each prepared urine, plasma, and liver sample were analyzed by HPLC. Column recoveries for urine were determined for each sample and ranged from 95.2 to 102% for Method A and from 80.8 to 98.3% for Method B. Due to carryover from previous samples, data from the Method B analysis for Female Animal M01640 are not reported. Column recoveries for plasma using Method A were determined on the first and last sample of the run and were 91.6 and 105%. Column recoveries for plasma using Method B were determined on each sample and ranged from 32.9 to 89.9%, with the exception of the 24-hour female sample for which the extraction recovery was 337%. All liver samples were analyzed using Method B, and column recoveries ranged from 102 to 128%. Liver samples from male Animal M01640 and female Animal M01644 were analyzed using Method A and column recoveries were 40.7 and 87.6%.

Method A

HPLC System

Pump:	HP 1050 Series for urine HP 1100 Series for plasma and liver
Autoinjector:	HP 1050 Series for urine HP 1100 Series for plasma and liver
Column heater:	HP 1050 Series for urine HP 1100 Series for plasma and liver
UV detector:	HP 1050 Series for urine HP 1100 Series for plasma and liver
Wavelength:	236 nm
Radioactivity detector:	Packard Radiomatic 500 Series
Flow cell:	0.5 mL TRLSC
Scintillation cocktail:	Ultima Flo M
Scintillation cocktail flow rate:	3 mL/minute
Column:	Zorbax SB-C18, 4.6 x 250 mm, 5µm
Guard column:	Phenomenex 4 x 3.0 mm C18 (plasma and liver only)
Column temperature:	25°C
Mobile phase A:	0.1% formic acid
Mobile phase B:	acetonitrile
Flow rate:	1 mL/minute

Gradient:

Time (Minutes)	A(%)	B(%)
Initial	98	2
5	98	2
30	30	70
31	98	2
40	98	2

Method B

HPLC System	
Pump:	HP 1100 Series
Autoinjector:	HP 1100 Series
Column heater:	HP 1100 Series
UV detector:	HP 1100 Series
Wavelength:	236 nm
Fraction collector:	ISCO Foxy 200
Radioactivity detector:	Packard Radiomatic 500 Series
Flow cell:	0.5 mL TRLSC
Scintillation cocktail:	Ultima Flo M
Scintillation cocktail flow rate:	3 mL/minute
Column:	Phenomenex Asahipak GPC GS-220HQ
Guard column:	Phenomenex Asahipak GPC GS-2G7B
Column temperature:	25°C
Mobile phase:	236.7 mM sodium phosphate, pH 3
Flow rate:	0.5 mL/minute (plasma samples, urine for LC/MS analysis, and urine glucuronidase samples)
	0.6 mL/minute (remaining urine and liver samples)

Metabolite Characterization

Selected urine and plasma samples were analyzed for ^{14}C-RDX and its metabolites by full-scan LC/MS with radiometric and UV detection for structural determination. Based on the molecular weight data obtained from the full-scan analyses, selected samples were analyzed using LC/MS/MS. The method is presented in Appendix 4.

Preparation of Urine for LC/MS Analysis

The pooled urine sample from male Animal M01638 was prepared for LC/MS analysis as follows. The pooled urine sample was placed in a tube and centrifuged for approximately 5 minutes. The supernatant was transferred to a new vial and duplicate weighed aliquots were taken and analyzed by LSC to determine radioactivity concentration. Multiple injections were made on the HPLC using Method B, and 30-second fractions were collected. Fractions from 15 to 32 minutes and from 42 to 52 minutes were pooled by time point and a single aliquot analyzed by LSC to determine the radioactivity concentration. The fractions from 43.5 to 45 minutes were combined and the fractions from 48.0 to 49.5 were combined. The two samples were then submitted for LC/MS analysis.

Preparation of Plasma for LC/MS Analysis

Individual plasma samples (1, 6, and 12 hours postdose) from male Animal M01638 and female Animal M01640 were prepared for analysis. To an aliquot of each sample (approximately 0.5 mL), approximately 1.5 mL of methanol was added, and the sample mixed on a vortex mixer. The sample was then centrifuged at approximately 20,817 rcf for approximately 10 minutes at approximately 5°C. The resulting supernatant was transferred to a new vial. The extraction was repeated using the sample procedure, and the supernatants combined. Each supernatant was concentrated under nitrogen at ambient temperature to approximately 0.5 mL. The concentrated samples were then filtered through a 0.45 µm Millipore filter unit and centrifuged at approximately 20,817 rcf for approximately 10 minutes at approximately 5°C. The filtrates were transferred to new vials and duplicate aliquots were analyzed by LSC to determine the concentration of radioactivity. A portion of each filtrate was analyzed by HPLC using Method A.

Disposition of Dose Formulations and Test Article

Any dose formulations remaining following dose administration were discarded. Any remaining test article (RDX and ^{14}C-RDX) will be maintained at Covance until either discarded or shipped to a site specified by the Sponsor. The remaining reference standards were shipped to the Sponsor on 23 August 2005.

Disposition of Raw Data, Records, Samples, and the Final Report

The original signed protocol, any amendments, the original signed report, the study correspondence, and raw data captured on durable media will be archived in the storage facilities of Covance. All other raw data, documentation, records, and specimens will be archived in the storage facilities of Covance until shipped to a site specified by the Sponsor (as described in Covance SOPs), as authorized by the Sponsor.

Data Analyses

Calculation methods and formulas are in Appendix 3. Statistical analyses were limited to simple expressions of variation, such as mean and standard deviation. Dose tables were compiled with mean and standard deviation values calculated using Excel, Version 8.0e (Microsoft Corporation). Data tables were generated by Debra, Version 5.2a (LabLogic Systems Ltd., Sheffield, UK). Debra is an automated and validated data capture and management system for data collection in absorption, distribution, and excretion studies using radiolabeled test article. Debra captures data from balances and scintillation counters. The percentages of radioactive dose in blood, muscle, and fat were calculated based on extrapolation values scaled to total body composition (Appendix 3).

RESULTS AND DISCUSSION

Test Article and Dose Formulation Analysis

Radiopurity and Stability

The radiopurity of ^{14}C-RDX was determined at Covance by HPLC to be 97.5% prior to dose preparation. The mean radiopurity values from HPLC analysis of predose and postdose aliquots were 95.3 and 96.1%, respectively. These data confirm stability of the

test article under conditions of the study. Representative stability chromatograms are presented in Appendix 2.

Concentration and Homogeneity
The LSC results indicate the dose was homogeneous during the dosing periods. Dose analysis results are presented in Appendix 2.

Specific Activity
The specific activity of ^{14}C-RDX as provided by the Sponsor was 39.20 mCi/mmol (177 μCi/mg). The specific activity of ^{14}C-RDX in the dose formulation was calculated by Covance to be 1.30 μCi/mg .

Acclimation
All animals appeared clinically healthy throughout acclimation with the following exception. Male pigs M01638 and M01640 managed to dislodge the divider between the cages and their inadvertent commingling developed into a scuffle. This resulted in superficial scrapes and abrasions to both pigs. A veterinary examination indicated no medical intervention was necessary. Animals were released from acclimation on 13 July 2004.

Body Weights and Doses Administered
Individual body and dose weights, as well as calculated dose and radioactivity administered, are presented in Table 1. Animals received an actual overall mean dose of 43.3 ±1.5 mg/kg.

Antemortem Observations
The following observations were noted following dose administration.

Animal Number	Observation
Male M01638	Vomitus was found at the 12-hour urine collection.
Male M01640	Excessive salivation at approximately 39 minutes postdose Vomited approximately 87 and 102 minutes postdose.
Female M01643	Whole body tremors approximately 9 minutes postdose. Vomited approximately 19 minutes postdose, and again approximately 21, 23, and 25 minutes postdose. Tremors again noted approximately 54 minutes postdose.
Female M01644	Vomited approximately 44 and 49 minutes postdose. Convulsions approximately 59 minutes postdose.

Symptoms associated with RDX appeared to subside approximately 2 to 2.5 hours postdose.

Distribution of Radioactivity in Blood, Plasma, and Tissues

The concentrations of radioactivity in blood and plasma at specified times postdose are presented in Table 2. The concentrations of radioactivity (μg equivalents ^{14}C-RDX/g) in selected tissues, the blood:plasma and tissue:plasma concentration ratios, and the percent of radioactive dose recovered in blood and selected tissues at specified times postdose are found in Tables 3 through 6. The blood and plasma concentration versus time profiles are presented graphically in Figures 1 and 2.

The concentrations of ^{14}C-RDX-derived radioactivity in blood and plasma reached maximum levels (C_{max}) at 12 hours postdose for both males and one female. The blood and plasma concentrations for the second female reached C_{max} at 6 hours postdose. In males, blood C_{max} values ranged from 7.18 to 14.4 μg equivalents ^{14}C-RDX/g and in females, values ranged from 6.49 to 7.90 μg equivalents ^{14}C-RDX/g. The plasma C_{max} values for males ranged from 8.76 to 18.3 μg equivalents ^{14}C-RDX/g, and for females values ranged from 8.54 to 10.0 μg equivalents ^{14}C-RDX/g. All animals vomited at various times and in varying amounts during the study. The data must therefore, be viewed with this in mind as this will have an effect on the variability in concentrations between animals.

The distribution of ^{14}C-RDX-derived radioactivity was extensive, with drug derived radioactivity quantifiable in all analyzed tissues at 24 hours postdose. In both males and females, the highest concentrations of ^{14}C-RDX-derived radioactivity were observed in liver, kidneys, and small intestine in all animals. The male and female average values for liver were 84.0 and 55.7 μg equivalents ^{14}C-RDX/g, respectively; for kidneys were 29.4 and 16.9 μg equivalents ^{14}C-RDX/g, respectively; and for small intestine were 16.0 and 10.0 μg equivalents ^{14}C-RDX/g, respectively. The lowest concentration values were observed in abdominal fat, skeletal muscle, and skin, with average values no higher than 5.67 μg equivalents ^{14}C-RDX/g.

Quantifiable levels of radioactivity in the brain and testes suggests ^{14}C-RDX-derived radioactivity crosses the blood/brain and blood/testes barriers.

The blood:plasma concentration ratios were less than one at all collection times for all animals. The tissue:plasma concentration ratios were greater than one for kidneys, liver, and small intestine, in all animals and less than one for brain, abdominal fat, heart, skeletal muscle, and skin in all animals. All remaining tissue:plasma concentration ratios showed interanimal variability, with some ratios greater than one and others less than one.

In males, the highest percentages of radioactive dose in tissues were observed in liver, skeletal muscle, stomach contents and wash, and abdominal fat, with average values of 5.80, 4.72, 3.75, and 2.79% of the administered dose, respectively. In females, the highest percentages in tissues were observed in liver and skeletal muscle, with average values of 3.48 and 2.47% of the administered dose, respectively.

Excretion
The amounts of radioactive dose in urine, feces, cage wash, cage wipe, and vomitus and the cumulative amounts of radioactive dose in urine and feces are presented in Tables 7 and 8, respectively. Elimination patterns are depicted graphically in Figures 3 and 4.

Following oral administration of ^{14}C-RDX, the major route of elimination of drug-derived radioactivity was via urine for both males and females. In males, at 24 hours postdose, urine and feces accounted for average values of 17.3 and 0.53% of the dosed radioactivity, respectively, and cage wash, cage wipe, and vomitus accounted for 0.42, 0.49, and 21.9% of the administered radioactivity, respectively. In females, urine and feces accounted for average values of 16.0 and 0.79% of the dosed radioactivity, respectively, and cage wash, cage wipe, and vomitus accounted for 0.17, 0.47, and 12.3% of the administered radioactivity, respectively. The overall average excretion recoveries in males and females were 40.6 and 29.7%, respectively.

The relatively low levels of radioactivity observed in gastrointestinal contents and wash as well as low levels in feces suggest nearly complete oral absorption of ^{14}C-RDX-derived radioactivity.

Metabolite Profiling and Characterization
Selected urine, plasma, and liver samples were profiled using two HPLC methods. Method A utilized a reverse phase column and was capable of separating the metabolite standards provided by the Sponsor. Method B was a mixed bed column which was a combination of reverse phase and size exclusion, with a molecular weight limit of <3000. Method B was not used to characterize the standards provided by the Sponsor, but was used to separate material eluting in the void volume of Method A. Selected samples of urine and plasma were also analyzed by LC/MS and LC/MS/MS.

The results of metabolic profiling in urine, expressed as percent of sample radioactivity and percent of dose, are presented in Tables 9 through 12. The results of profiling in plasma and liver, expressed as percent of sample radioactivity and concentration (μg equivalents ^{14}C-RDX/g), are presented in Tables 13 through 20. Chromatograms of reference substances and radiochromatograms are shown in Figures 5 through 24. The proposed metabolic pathway is presented in Figure 25. LC/MS results are presented in Appendix 4.

Urine Metabolite Profiling
Three metabolites were identified in minipig urine using HPLC Methods A and B. Profiling of the 0- to 24-hour pooled urine showed metabolites 4-nitro-2,4-diazabutanal (M1), 4-nitro-2,4-diaza-butanamide (M2), and parent RDX. HPLC Method A showed quantifiable levels of RDX representing from 1.02 to 6.32% of the sample radioactivity, and 0.11 to 0.87% of the dosed radioactivity. Analysis using Method B showed two metabolites which did not elute in Method A. These metabolites were isolated and submitted for LC/MS analysis. Mass spectrometry analysis showed that these metabolites, designated as M1 and M2, result from the opening of the RDX ring. M1 represented 5.81 to 12.23% of the sample radioactivity and 0.97 to 2.48% of the dosed

radioactivity. M2 represented 2.27 to 3.93% of the sample radioactivity or 0.35 to 0.88% of the radioactive dose. Most of the profiled radioactivity was not identified and was observed at or near the void volume in methods using reverse phase columns and a mixed phase column utilizing reverse phase and size exclusion. This suggests that most of the radioactivity is very water-soluble and of higher molecular weight.

Glucuronidase/sulfatase hydrolysis of a urine sample showed no notable change in the HPLC profile using Method B, suggesting that no glucuronide or sulfate conjugates were present.

Analysis by LC/MS showed trace amounts of the RDX metabolites MNX and DNX in male urine and MNX in female urine; however, no quantifiable levels of radioactivity were observed.

Plasma Metabolite Profiling

Profiling of selected plasma samples collected at 1, 6, 12, and 24 hours postdose, using Method A, showed the parent RDX and 2 unknowns eluting at or near the void volume. In males, from 1 to 12 hours postdose, RDX represented 67.8 to 79.5% of the sample radioactivity and 4.35 to 7.60 μg equivalents ^{14}C-RDX/g. At 24 hours postdose, RDX represented over 47% of the sample radioactivity, corresponding to 3.33 μg equivalents ^{14}C-RDX/g. In females, at 1, 6, 12, and 24 hours postdose, RDX represented 83.0, 55.1, 46.2, and 10.0% of the sample radioactivity, and 3.40, 3.35, 2.64, and 0.336 μg equivalents ^{14}C-RDX/g, respectively. Analysis of plasma using HPLC Method B showed no M1 or M2 metabolites at any time point.

Trace amounts of RDX metabolites MNX, DNX, and TNX were observed in plasma by LC/MS; however, no quantifiable levels of radioactivity were observed.

Liver Metabolite Profiling

Profiling of selected liver samples by HPLC (Methods A and B) showed only two unknown peaks and no parent RDX, M1, or M2 metabolites.

LC/MS/MS Analysis of RDX in Brain and Liver

The results and method for analysis of RDX in brain and liver are presented in Appendix 5.

Quantitative analysis of RDX in liver and brain at 24 hours postdose was performed by LC/MS/MS. Concentrations of RDX in the brains ranged from 33.5 to 200 ng/g in females and 419 to 1070 ng/g in males. RDX concentrations in liver were less than 1 ng/g.

CONCLUSIONS

Following oral administration of ^{14}C-RDX to Yucatan minipigs, distribution of ^{14}C-RDX-derived radioactivity was extensive, with drug derived radioactivity quantifiable in all analyzed tissues at 24 hours postdose.

Quantifiable levels of radioactivity in the brain and testes suggests ^{14}C-RDX-derived radioactivity crosses the blood/brain and blood/testes barriers.

Urine was the primary route of elimination of radioactivity following oral administration of ^{14}C-RDX.

Relatively low levels of radioactivity observed in the gastrointestinal contents and wash as well as low levels in the feces suggest nearly complete oral absorption of ^{14}C-RDX-derived radioactivity.

Following oral administration of ^{14}C-RDX, the parent RDX and metabolites M1 and M2 were identified in urine by LC/MS.

Trace amounts of RDX metabolites MNX, DNX, and TNX were observed in plasma by LC/MS; however, no quantifiable levels of radioactivity were observed.

LC/MS/MS analysis of RDX showed concentrations in brain ranged from 33.5 to 200 ng/g in females and 419 to 1070 ng/g in males. RDX concentrations in liver were less than 1 ng/g.

TABLES

Table 1
Individual body weights of and radioactive doses administered to male and female minipigs dosed orally with ^{14}C-RDX (45 mg/kg)

Animal Number	Body Weight (kg)	Dose Weight (g)	Dose Wipe (dpm)	Dose Administered (dpm)	(mg/animal)	(mg/kg)	(µCi/animal)	(µCi/kg)
				Males				
M01638	16.4	80.7422	19673076	2086377364	725	44.2	940	57.3
M01640	15.4	75.4813	23553280	1950436672	677	44.0	879	57.1
Average					701	44.1	910	57.2
				Females				
M01643	14.7	71.8313	16816678	1856120786	645	43.9	836	56.9
M01644	15.7	71.6837	151253482	1852307766	643	41.0	834	53.1
Average					644	42.5	835	55.0
Overall Mean					673	43.3	872	56.1
Overall SD					38	1.5	50	2.0

SD Standard deviation.

Note: Dose concentration was 25,800,000 dpm/g and the specific activity of the ^{14}C-RDX was 1.30 µCi/mg.

Table 2
Concentrations of radioactivity in blood and plasma at specified times after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs

Collection Time (Hours)	µg Equivalents ^{14}C-RDX/g Animal Number					
	Males			Females		
	M01638	M01640	Average	M01643	M01644	Average
			Blood			
1	4.26	3.51	3.88	5.26	3.38	4.32
6	8.60	6.29	7.45	6.49	6.13	6.31
12	14.4	7.18	10.8	5.00	7.90	6.45
24	12.7	6.28	9.49	3.51	7.27	5.39
			Plasma			
1	5.67	4.71	5.19	7.31	4.64	5.98
6	11.5	8.28	9.91	8.54	8.53	8.53
12	18.3	8.76	13.6	6.12	10.0	8.07
24	15.3	7.61	11.4	6.99	8.52	7.76

Table 3
Concentrations of radioactivity in selected tissues at 24 hours postdose after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs

	µg Equivalents ^{14}C-RDX/g					
	Animal Number					
	Males			Females		
Sample	M01638	M01640	Average	M01643	M01644	Average
Blood	12.7	6.28	9.49	3.51	7.27	5.39
Brain	10.7	5.19	7.93	2.60	5.59	4.10
Fat (abdominal)	7.76	3.38	5.57	1.56	3.26	2.41
Heart	12.1	6.11	9.12	3.43	6.96	5.19
Kidneys	37.3	21.4	29.4	9.99	23.8	16.9
Large intestine	15.1	7.90	11.5	5.11	8.59	6.85
Liver	106	62.4	84.0	43.4	67.9	55.7
Lungs	15.3	8.48	11.9	4.27	9.08	6.67
Muscle (skeletal)	7.71	3.64	5.67	1.90	3.74	2.82
Ovaries	NA	NA	NA	4.56	9.40	6.98
Plasma	15.3	7.61	11.4	6.99	8.52	7.76
Skin	6.41	3.16	4.78	1.17	2.93	2.05
Small intestine	20.4	11.7	16.0	7.02	13.1	10.0
Stomach	18.8	10.4	14.6	3.71	8.82	6.26
Testes	14.0	8.76	11.4	NA	NA	NA

NA Not applicable.

Table 4
Blood:plasma concentration ratios at specified times after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs

Collection Time (Hours)	Blood:Plasma Concentration Ratios					
	Animal Number					
	Males			Females		
	M01638	M01640	Average	M01643	M01644	Average
1	0.751	0.745	0.748	0.719	0.729	0.724
6	0.746	0.760	0.753	0.760	0.719	0.739
12	0.785	0.820	0.802	0.816	0.789	0.803
24	0.831	0.826	0.829	0.502	0.853	0.677

Table 5
Tissue:plasma concentration ratios at 24 hours postdose after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs

	Tissue:Plasma Concentration Ratios					
	Animal Number					
	Males			Females		
Sample	M01638	M01640	Average	M01643	M01644	Average
Brain	0.698	0.683	0.690	0.372	0.656	0.514
Fat (abdominal)	0.508	0.444	0.476	0.223	0.382	0.303
Heart	0.794	0.804	0.799	0.490	0.816	0.653
Kidneys	2.44	2.82	2.63	1.43	2.80	2.11
Large intestine	0.989	1.04	1.01	0.730	1.01	0.869
Liver	6.91	8.21	7.56	6.21	7.98	7.09
Lungs	0.999	1.12	1.06	0.610	1.07	0.838
Muscle (skeletal)	0.505	0.478	0.492	0.271	0.439	0.355
Ovaries	NA	NA	NA	0.652	1.10	0.878
Skin	0.420	0.415	0.417	0.168	0.344	0.256
Small intestine	1.34	1.54	1.44	1.00	1.53	1.27
Stomach	1.23	1.36	1.30	0.531	1.04	0.783
Testes	0.920	1.15	1.04	NA	NA	NA

NA Not applicable.

Table 6
Percent of radioactive dose in selected tissues at 24 hours postdose after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs

	Percent of Radioactive Dose					
	Animal Number					
	Males			Females		
Sample	M01638	M01640	Average	M01643	M01644	Average
Blood	1.72	0.86	1.29	0.48	1.07	0.78
Brain	0.10	0.06	0.08	0.03	0.06	0.05
Fat (abdominal)	3.88	1.70	2.79	0.79	1.76	1.27
Heart	0.14	0.07	0.11	0.04	0.09	0.07
Kidneys	0.38	0.21	0.30	0.10	0.22	0.16
Large intestine	0.75	0.31	0.53	0.24	0.39	0.32
Large intestine contents and wash	1.51	1.08	1.30	0.38	1.13	0.76
Liver	7.85	3.74	5.80	2.87	4.09	3.48
Lungs	0.26	0.15	0.21	0.07	0.15	0.11
Muscle (skeletal)	6.41	3.03	4.72	1.59	3.35	2.47
Ovaries	NA	NA	NA	0.00	0.01	0.01
Skin	0.03	0.01	0.02	0.01	0.02	0.02
Small intestine	1.47	0.80	1.14	0.46	0.84	0.65
Small intestine contents and wash	0.54	0.38	0.46	0.10	0.21	0.16
Stomach	0.44	0.26	0.35	0.09	0.19	0.14
Stomach contents and wash	4.23	3.27	3.75	0.01	0.20	0.11
Testes	0.06	0.03	0.05	NA	NA	NA
Total	29.8	16.0	22.9	7.26	13.8	10.5

NA Not applicable.

Note: Blood, fat, and muscle are extrapolated per body weight for percent of radioactive dose.

Table 7
Percent of radioactive dose in urine, feces, cage wash, cage wipe, and vomitus at specified intervals after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs

Collection Interval (Hours)	Percent of Radioactive Dose					
	Animal Number					
	Males			Females		
	M01638	M01640	Average	M01643	M01644	Average
			Urine			
0-6	2.60	1.66	2.13	6.86	3.91	5.39
6-12	8.58	4.93	6.76	4.37	6.79	5.58
12-24	12.2	4.53	8.37	3.11	6.87	4.99
Subtotal	23.4	11.1	17.3	14.3	17.6	16.0
			Feces			
0-24	0.65	0.40	0.53	0.63	0.94	0.79
Subtotal	0.65	0.40	0.53	0.63	0.94	0.79
			Cage Wash, Cage Wipe, and Vomitus			
24[a]	0.52	0.32	0.42	0.13	0.20	0.17
24[b]	0.11	0.86	0.49	0.39	0.54	0.47
12[c]	0.80	43.0	21.9	14.8	9.74	12.3
Total	25.5	55.7	40.6	30.3	29.0	29.7

a Cage wash.
b Cage wipe.
c Vomitus.

Table 8
Cumulative percent of radioactive dose in urine and feces at specified intervals after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs

Collection Interval (Hours)	Percent of Radioactive Dose					
	Animal Number					
	Males			Females		
	M01638	M01640	Average	M01643	M01644	Average
			Urine			
0-6	2.60	1.66	2.13	6.86	3.91	5.39
0-12	11.2	6.59	8.90	11.2	10.7	11.0
0-24	23.4	11.1	17.3	14.3	17.6	16.0
			Feces			
0-24	0.65	0.40	0.53	0.63	0.94	0.79

Table 9
Percent of sample radioactivity as ^{14}C-RDX or metabolites of ^{14}C-RDX in pooled urine (0-24 hours postdose) after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs (Method A)

Peak	Retention Time (Minutes)	Proposed Identification	Percent of Sample Radioactivity			
			Males		Females	
			M01638	M01640	M01643	M01644
1	3.4	Unknown 1	57.20	52.13	68.63	73.00
2	5.1-5.2	Unknown 2	10.34	11.79	4.42	9.32
3	5.4-6.0	Unknown 3	15.07	13.32	18.74	3.30
Region 1	9.4-23.6		16.01	21.60	1.89	12.89
Parent	25.4-25.5	RDX	1.02	1.02	6.32	1.24
		Total	99.6	99.9	100	99.8

Note: Trace levels of MNX and DNX were detected in male urine and MNX in female urine; however, no quantifiable levels of radioactivity were noted.

Table 10
Percent of radioactive dose as ^{14}C-RDX or metabolites of ^{14}C-RDX in pooled urine (0-24 hours postdose) after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs (Method A)

Peak	Retention Time (Minutes)	Proposed Identification	Percent of Radioactive Dose			
			Males		Females	
			M01638	M01640	M01643	M01644
1	3.4	Unknown 1	12.8	5.64	9.47	12.2
2	5.1-5.2	Unknown 2	2.31	1.28	0.61	1.56
3	5.4-6.0	Unknown 3	3.36	1.44	2.59	0.55
Region 1	9.4-23.6		3.57	2.34	0.26	2.16
Parent	25.4-25.5	RDX	0.23	0.11	0.87	0.21
		% of dose quantitated:	22.3	10.8	13.8	16.7
		% of dose excreted (0-24 hours):	23.4	11.1	14.3	17.6

Note: Trace levels of MNX and DNX were detected in male urine and MNX in female urine; however, no quantifiable levels of radioactivity were noted.

Table 11
Percent of sample radioactivity as ^{14}C-RDX or metabolites of ^{14}C-RDX in pooled urine (0-24 hours postdose) after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs (Method B)

Peak	Retention Time (Minutes)	Proposed Identification	Percent of Sample Radioactivity		
			Males		Female
			M01638	M01640	M01644
1	13.9-42.3	Unknown 1	84.86	81.80	91.76
M1	43.9-44.5	4-nitro-2,4-diazabutanal	11.13	12.23	5.81
M2	49.10	4-nitro-2,4-diaza-butanamide	3.93	3.24	2.27
		Total	99.9	97.3	99.8

Note: Due to carryover from previous samples, data from female Animal M01640 are not reported.

Trace levels of MNX and DNX were detected in male urine and MNX in female urine; however, no quantifiable levels of radioactivity were noted.

Table 12
Percent of radioactive dose as ^{14}C-RDX or metabolites of ^{14}C-RDX in pooled urine (0-24 hours postdose) after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs (Method B)

Peak	Retention Time (Minutes)	Proposed Identification	Percent of Radioactive Dose		
			Males		Female
			M01638	M01640	M01644
1	13.9-42.3	Unknown 1	18.9	8.85	15.4
M1	43.9-44.5	4-nitro-2,4-diazabutanal	2.48	1.32	0.97
M2	49.10	4-nitro-2,4-diaza-butanamide	0.88	0.35	0.38
		% of dose quantitated:	22.3	10.5	16.8
		% of dose excreted (0-24 hours):	23.4	11.1	17.6

Note: Due to carryover from previous samples, data from female Animal M01640 are not reported.

Trace levels of MNX and DNX were detected in male urine and MNX in female urine; however, no quantifiable levels of radioactivity were noted.

Table 13
Percent of sample radioactivity as ^{14}C-RDX or metabolites of ^{14}C-RDX in plasma at 1, 6, 12, and 24 hours after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs (Method A)

			Percent of Sample Radioactivity							
	Retention Time		Collection Time (Hours)							
			Male M01638				Female M01644			
Peak	(Minutes)	Proposed Identification	1	6	12	24	1	6	12	24
1	2.3-3.0	Unknown 1	7.23	20.33	21.35	40.40	9.43	29.49	44.23	73.33
2	3.9-5.0	Unknown 2	13.25	7.14	9.74	9.09	7.55	15.38	9.62	16.67
Parent	25.40	RDX	79.52	71.98	67.79	47.47	83.02	55.13	46.15	10.00
		Total	100	99.5	98.9	97.0	100	100	100	100

Note: Trace levels of MNX, DNX, and TNX were detected in the samples by LC/MS; however no quantifiable levels of radioactivity were noted.

Table 14
Concentrations of radioactivity as ^{14}C-RDX or metabolites of ^{14}C-RDX in plasma at 1, 6, 12, and 24 hours after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs (Method A)

			µg Equivalents ^{14}C-RDX/g							
	Retention Time		Collection Time (Hours)							
			Male M01638				Female M01644			
Peak	(Minutes)	Proposed Identification	1	6	12	24	1	6	12	24
1	2.3-3.0	Unknown 1	0.395	1.86	2.40	2.84	0.386	1.79	2.53	2.46
2	3.9-5.0	Unknown 2	0.724	0.652	1.09	0.638	0.309	0.935	0.551	0.560
Parent	25.4	RDX	4.35	6.57	7.60	3.33	3.40	3.35	2.64	0.336
		µg equivalents/g of dose identified:	5.47	9.08	11.1	6.81	4.10	6.08	5.72	3.36
		µg equivalents/g in sample:	5.67	11.5	18.3	15.3	4.64	8.53	10.0	8.52

Note: Trace levels of MNX, DNX, and TNX were detected in the samples by LC/MS; however no quantifiable levels of radioactivity were noted.

Table 15
Percent of sample radioactivity as ^{14}C-RDX or metabolites of ^{14}C-RDX in plasma at 1, 6, 12, and 24 hours after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs (Method B)

Peak	Retention Time (Minutes)	Proposed Identification	Percent of Sample Radioactivity							
			Collection Time (Hours)							
			Male M01638				Female M01644			
			1	6	12	24	1	6	12	24
Region 1	11.4-12.6		ND	61.05	58.21	73.02	ND	27.50	62.97	46.76
Region 2	17.9-19.7		ND	22.38	8.07	6.12	ND	31.25	6.65	7.19
Region 3	22.7-23.3		ND	11.92	23.92	12.24	ND	26.25	28.80	46.04
		Total	ND	95.4	90.2	91.4	ND	85.0	98.4	100

ND Not detected.

Note: Trace levels of MNX, DNX, and TNX were detected in the samples by LC/MS; however no quantifiable levels of radioactivity were noted.

Table 16
Concentrations of radioactivity as ^{14}C-RDX or metabolites of ^{14}C-RDX in plasma at 1, 6, 12, and 24 hours after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs (Method B)

Peak	Retention Time (Minutes)	Proposed Identification	µg Equivalents ^{14}C-RDX/g							
			Collection Time (Hours)							
			Male M01638				Female M01644			
			1	6	12	24	1	6	12	24
Region 1	11.4-12.6		ND	5.29	5.52	6.02	ND	1.92	11.9	3.43
Region 2	17.9-19.7		ND	1.94	0.765	0.505	ND	2.19	1.25	0.527
Region 3	22.7-23.3		ND	1.03	2.27	1.01	ND	1.84	5.43	3.37
		µg equivalents/g of dose identified:	ND	8.26	8.56	7.54	ND	5.95	18.6	7.33
		µg equivalents/g in sample:	4.64	8.53	10.0	8.52	5.67	11.5	18.3	15.3

ND Not detected.

Note: Trace levels of MNX, DNX, and TNX were detected in the samples by LC/MS; however no quantifiable levels of radioactivity were noted.

Table 17
Percent of sample radioactivity as ^{14}C-RDX or metabolites of ^{14}C-RDX in 24-hour liver samples after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs (Method A)

Peak	Retention Time (Minutes)	Proposed Identification	Percent of Sample Radioactivity	
			Male M01640	Female M01644
1	2.80	Unknown 1	98.46	97.51
2	4.2-4.9	Unknown 2	0.93	1.28
		Total	99.4	98.8

Table 18
Concentrations of radioactivity as ^{14}C-RDX or metabolites of ^{14}C-RDX in 24-hour liver samples after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs (Method A)

Peak	Retention Time (Minutes)	Proposed Identification	Percent of Sample Radioactivity	
			Male M01640	Female M01644
1	2.80	Unknown 1	25.8	27.5
2	4.2-4.9	Unknown 2	0.244	0.362
		µg equivalents/g of dose identified:	26.0	27.9
		µg equivalents/g in sample:	62.4	67.9

Table 19
Percent of sample radioactivity as ^{14}C-RDX or metabolites of ^{14}C-RDX in 24-hour liver samples after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs (Method B)

Peak	Retention Time (Minutes)	Proposed Identification	Percent of Sample Radioactivity			
			Males		Females	
			M01638	M01640	M01643	M01644
1	16.3-28.8	Unknown 1	100.00	100.00	100.00	100.00
		Total	100	100	100	100

Table 20
Concentrations of radioactivity as ^{14}C-RDX or metabolites of ^{14}C-RDX in 24-hour liver samples after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male and female minipigs (Method B)

Peak	Retention Time (Minutes)	Proposed Identification	µg Equivalents ^{14}C-RDX/g			
			Males		Females	
			M01638	M01640	M01643	M01644
1	16.3-28.8	Unknown 1	58.5	26.2	21.7	28.2
		µg equivalents/g of dose identified:	58.5	26.2	21.7	28.2
		µg equivalents/g in sample:	106	62.4	43.4	67.9

Table 21
Concentrations of RDX in the Brain and Liver following LC/MS/MS Analysis

Animal Number	Sex	Time (Hour)	Concentration (ng RDX/g)
		Brain	
M01638	M	24	1070
M01640	M	24	419
M01643	F	24	33.5
M01644	F	24	200
		Liver	
M01638	M	24	BLQ
M01640	M	24	BLQ
M01643	F	24	BLQ
M01644	F	24	BLQ

BLQ Below limit of quantitation.
F Female.
M Male.

FIGURES

Figure 1
Concentrations of radioactivity in blood and plasma at specified times after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male minipigs

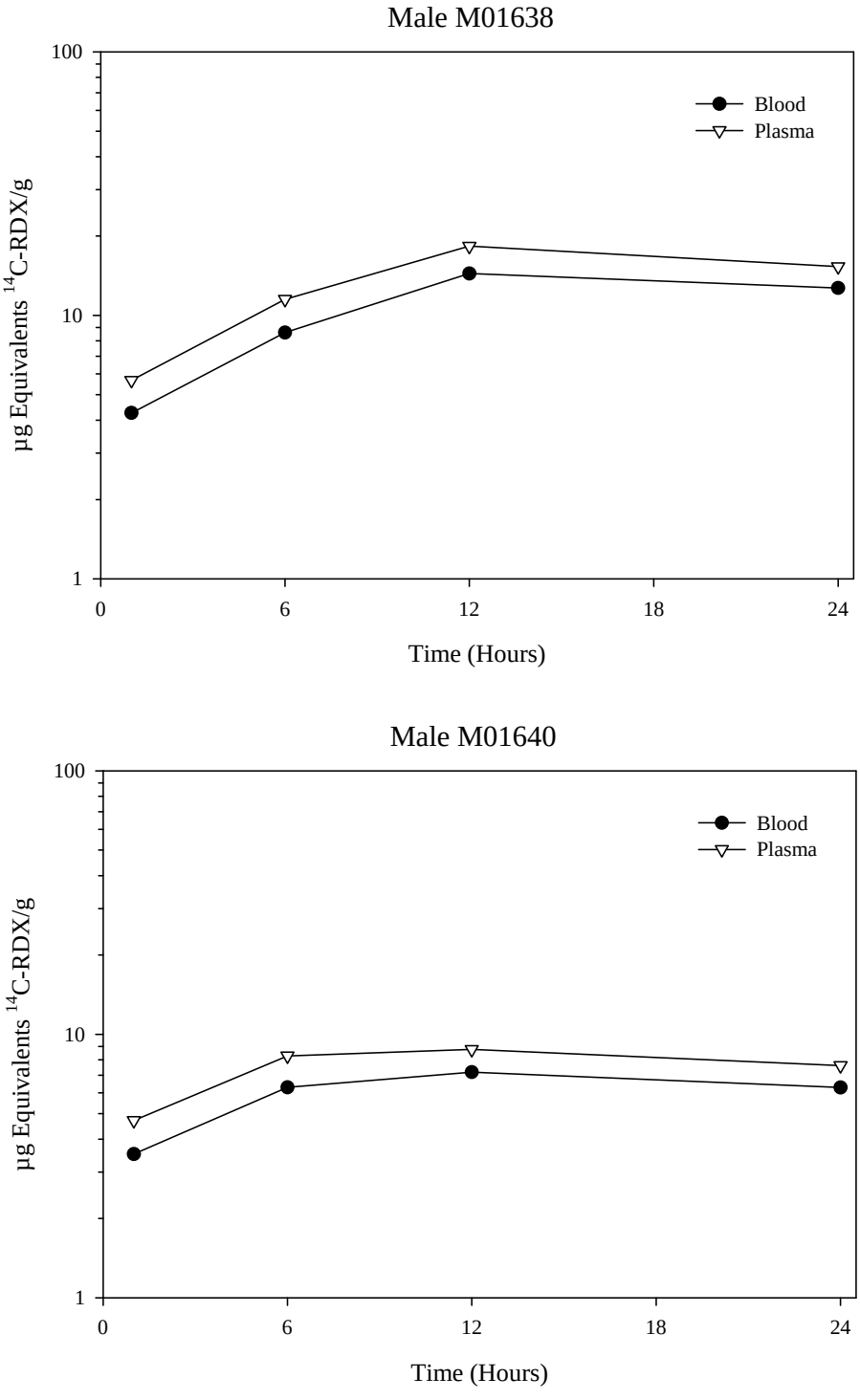
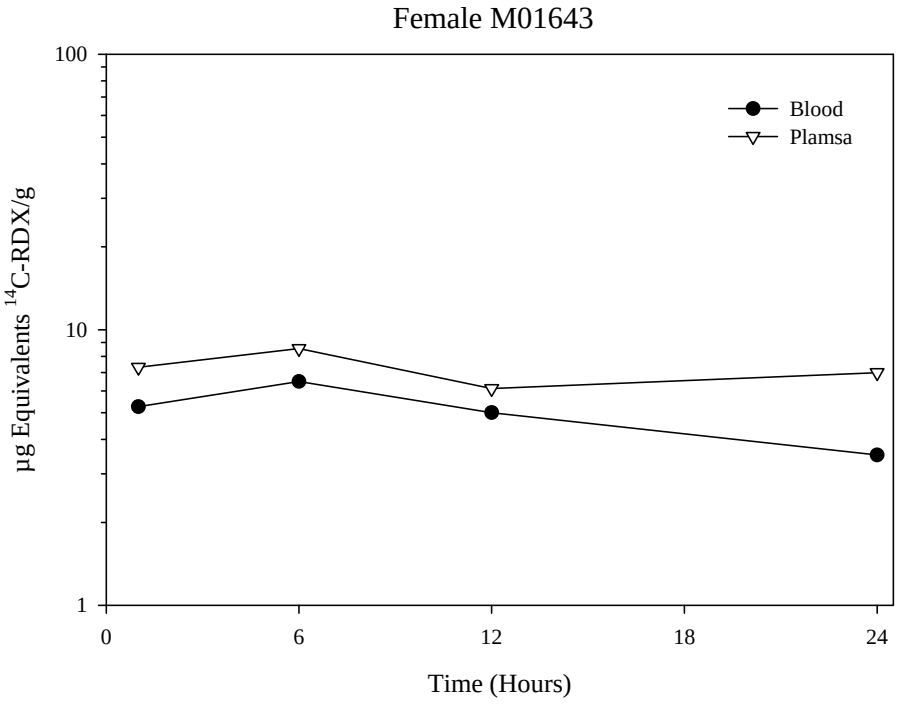


Figure 2
Concentrations of radioactivity in blood and plasma at specified times after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to female minipigs



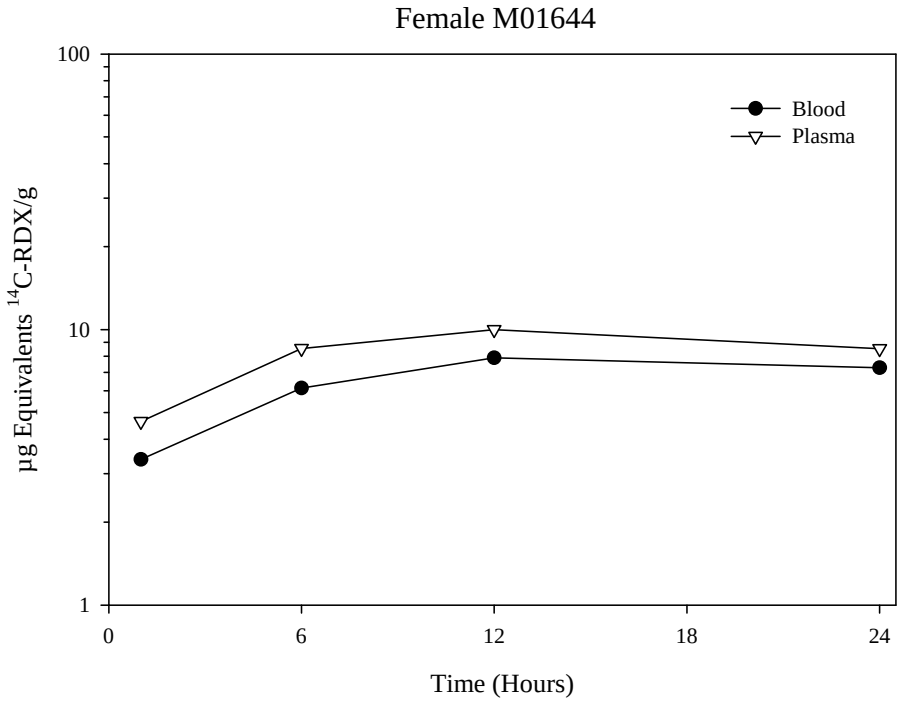
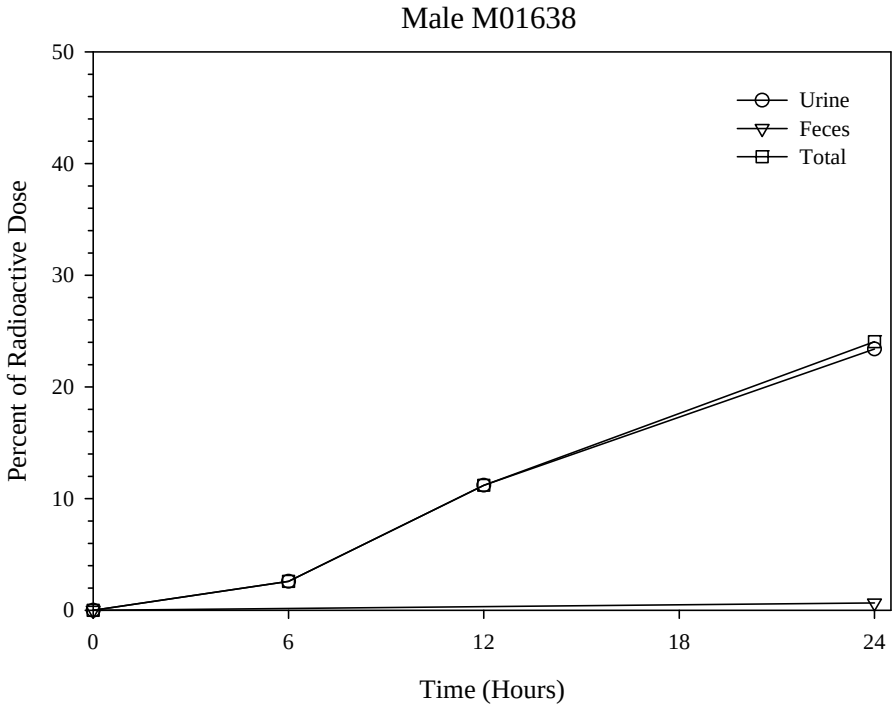


Figure 3
Cumulative percent of radioactive dose in urine and feces at specified intervals after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to male minipigs



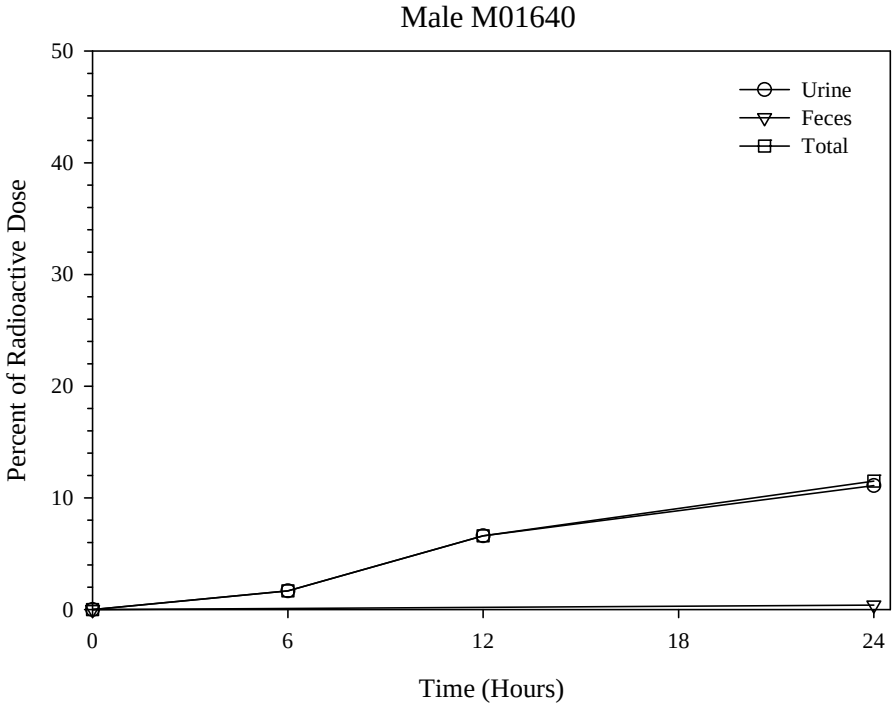
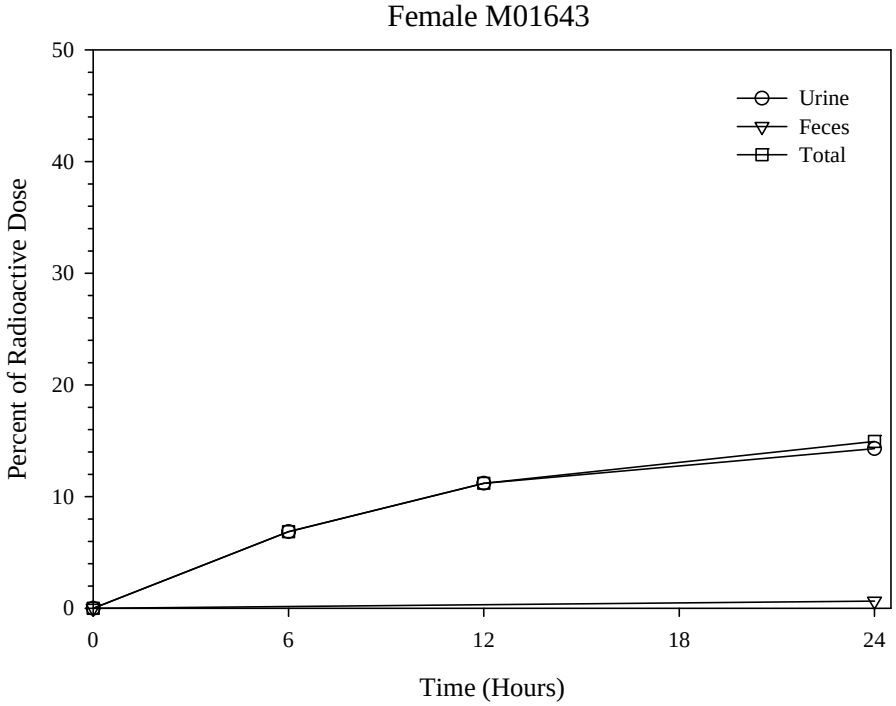


Figure 4
Cumulative percent of radioactive dose in urine and feces at specified intervals after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) to female minipigs



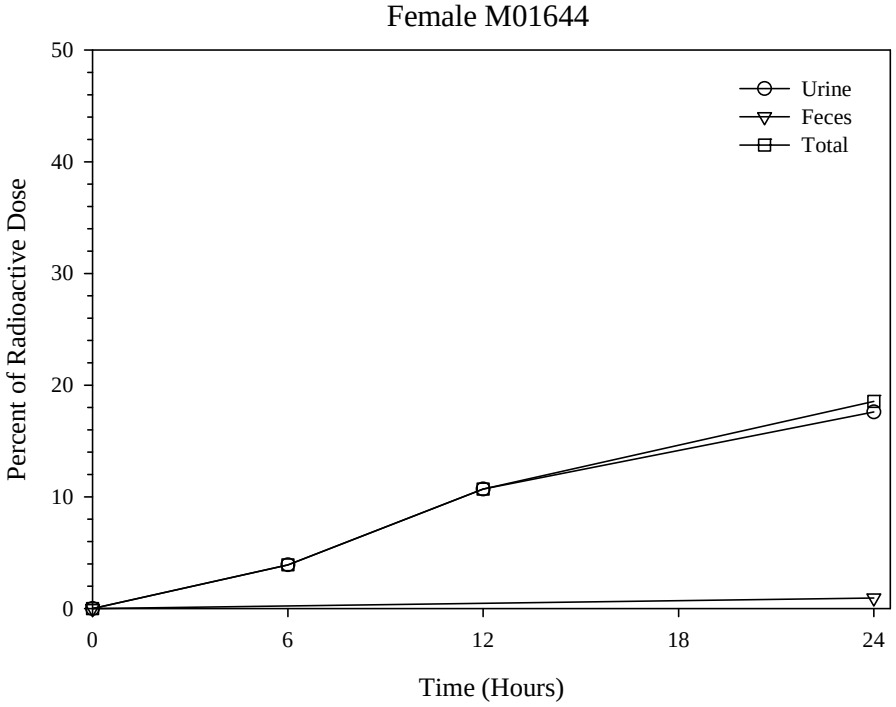
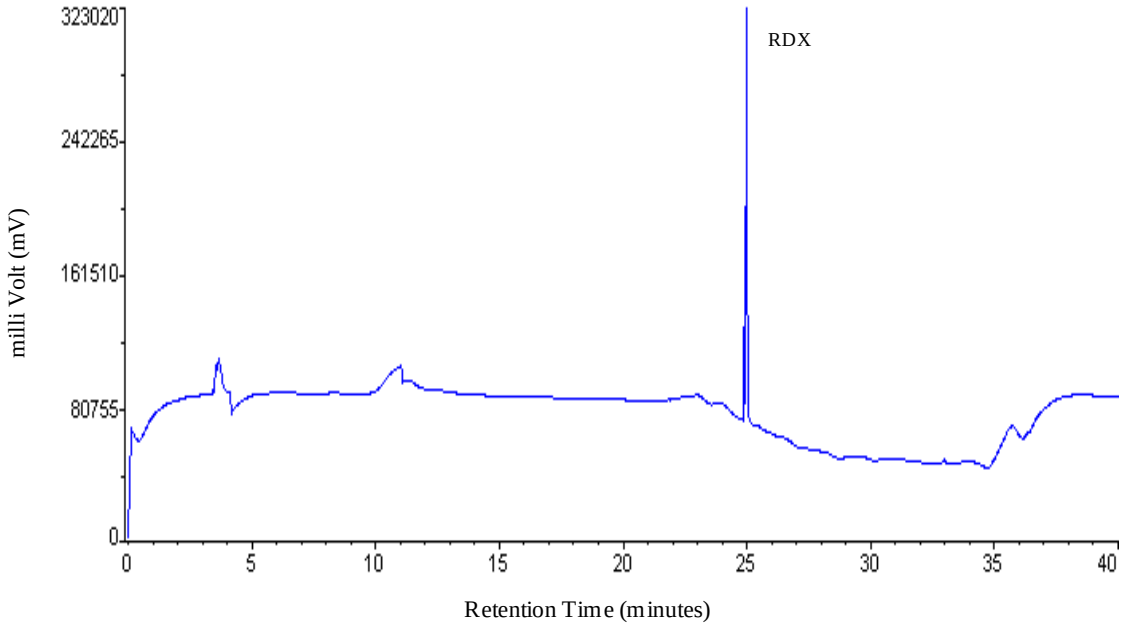


Figure 5
UV chromatograms of RDX and TNX reference substance (Method A)

Cold Standard – RDX (Cyclotrimethylenetrinitramine)



Cold Standard – TNX (1,3,5 trinitro-1,3,5-trazacyclohexane)

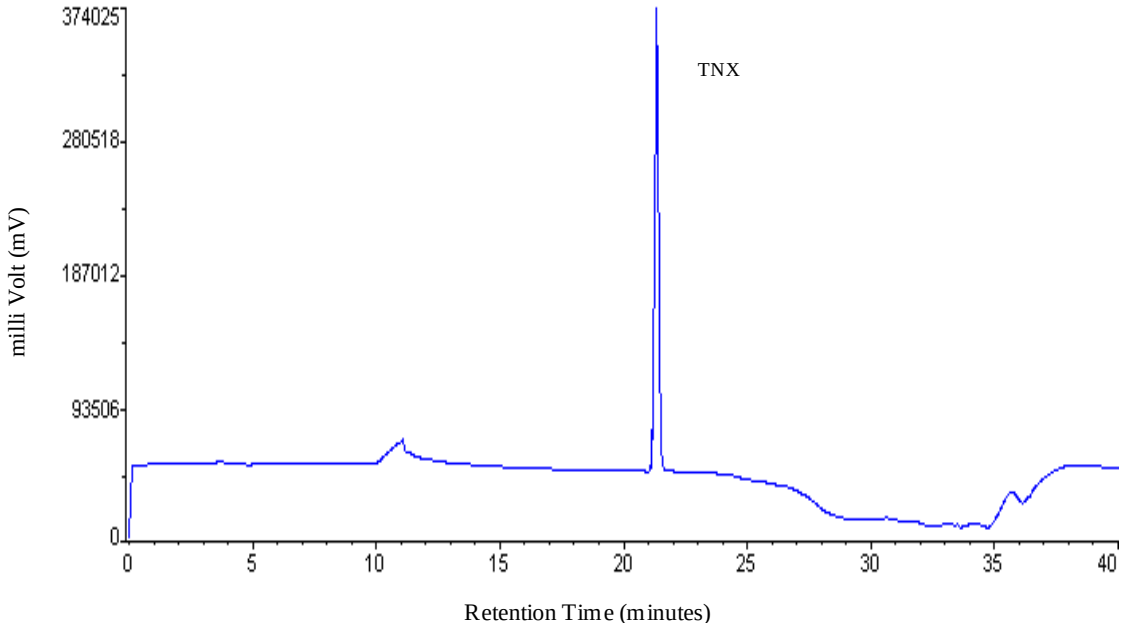
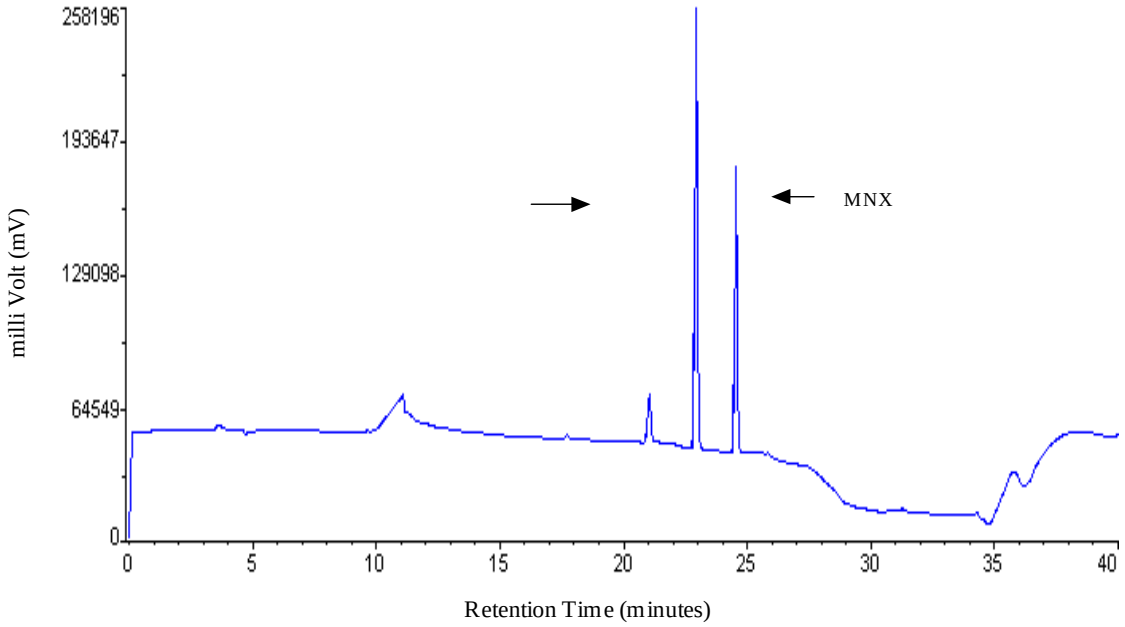


Figure 6
UV chromatograms of MNX and DNX reference substances (Method A)

Cold Standard – MNX (1-nitroso-3,5-dinitro-1,3,5-triazacyclohexane)



Cold Standard – DNX (1-nitro-3,5-dinitroso-1,3,5-triazacyclohexane)

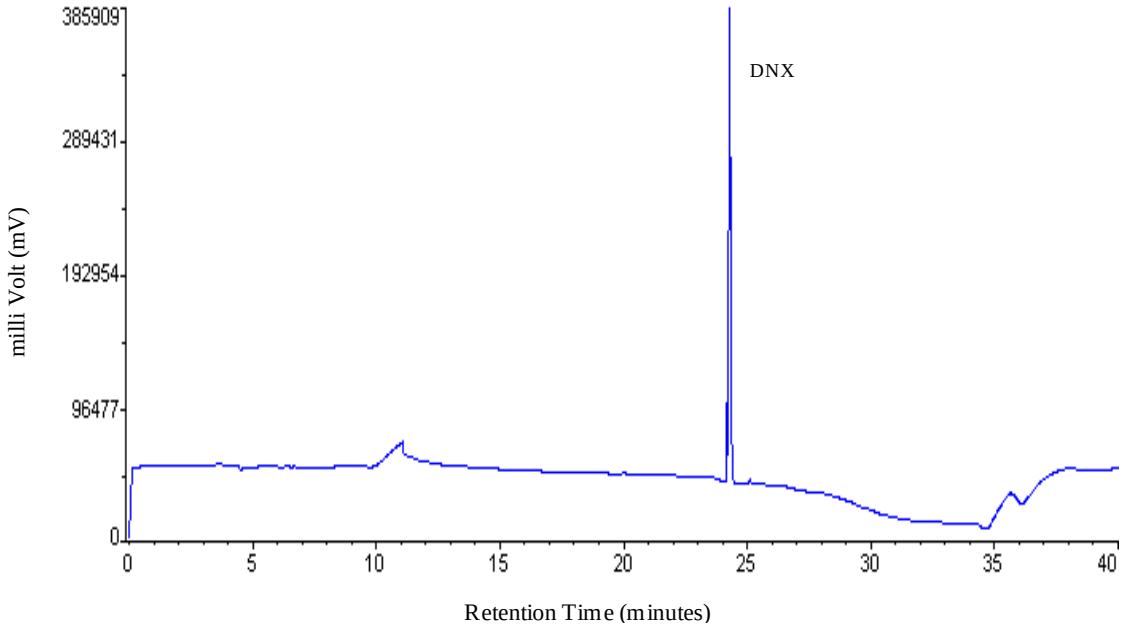
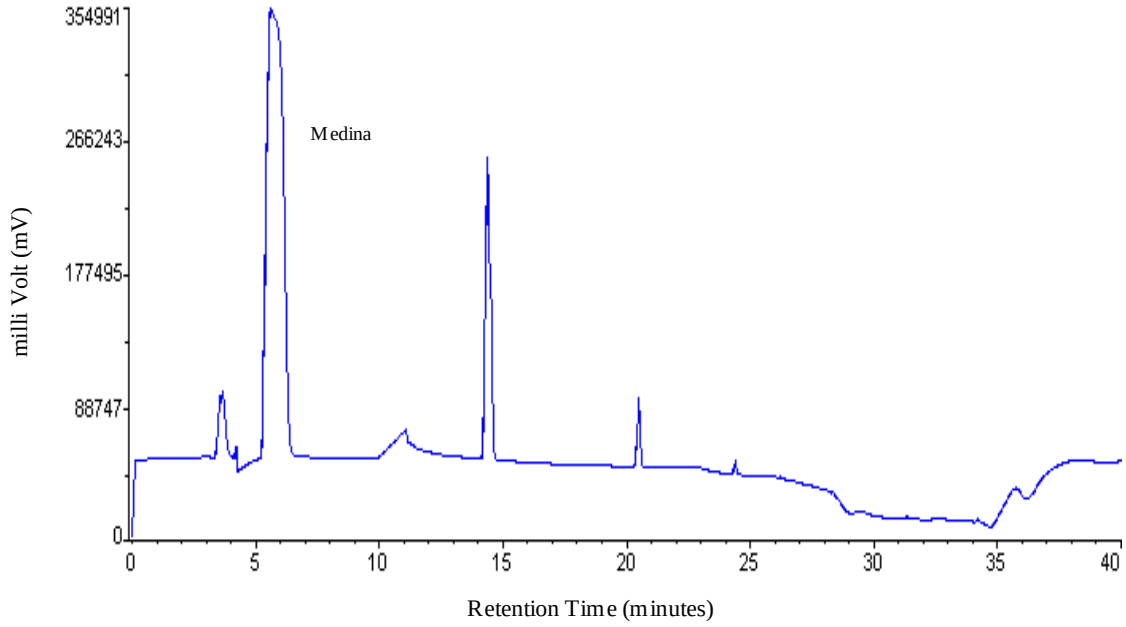


Figure 7
UV chromatograms of MEDINA and 4-nitro-2,4-diazabutanal reference substances (Method A)

Cold Standard – Medina (methylenedinitramine)



Cold Standard – 4-nitro-2,4-diazabutanal

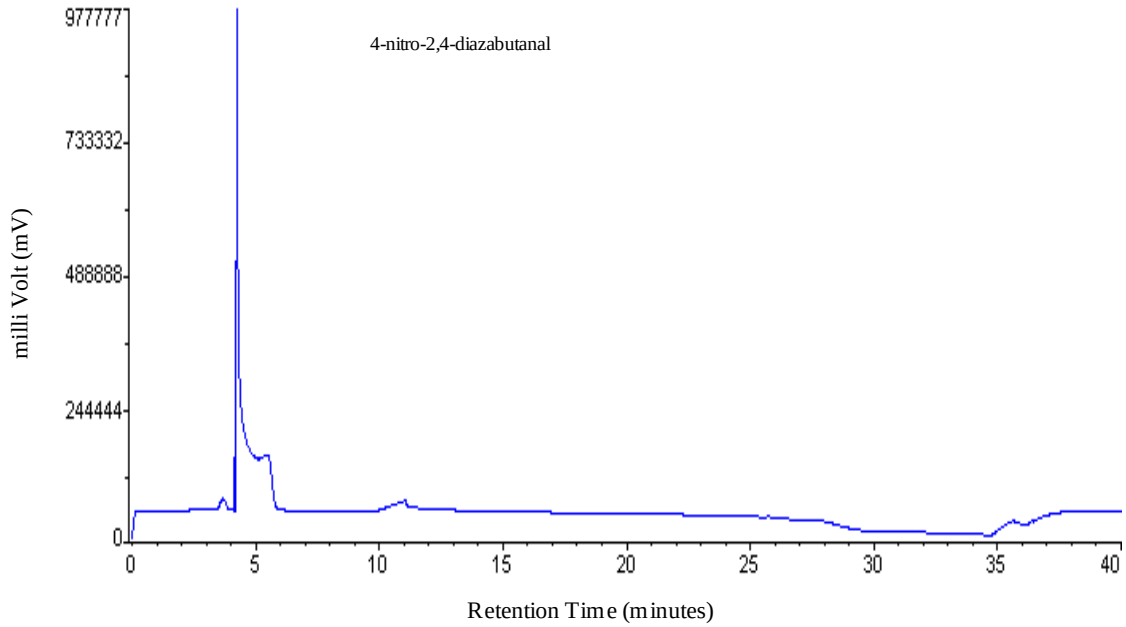
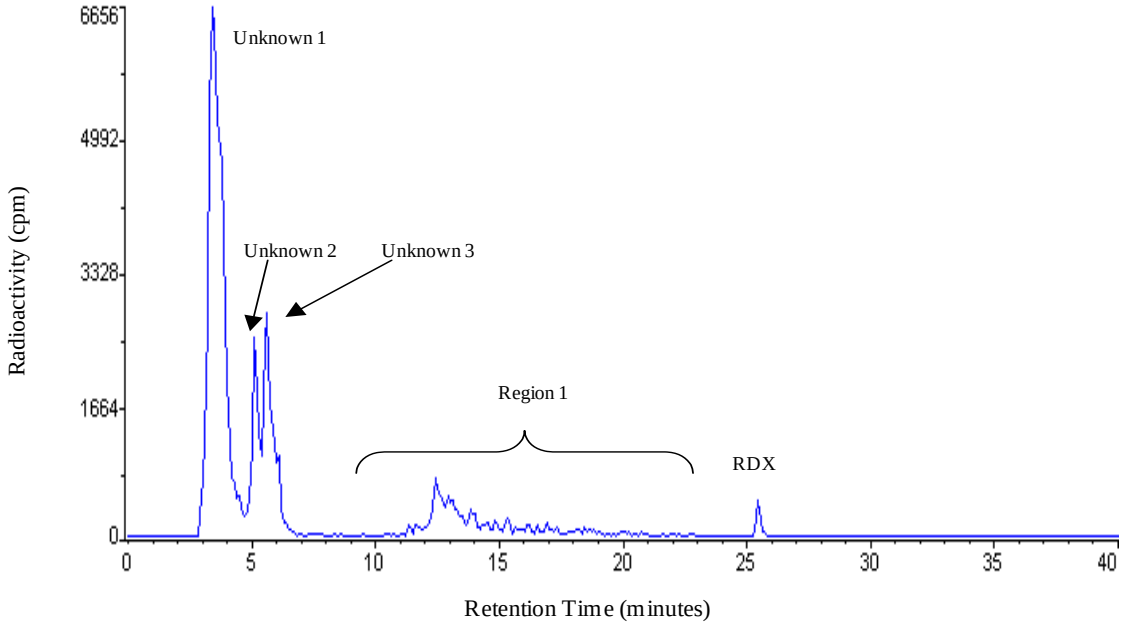


Figure 8
Radiochromatograms of pooled urine (0-24 hours) from male Animals M01638 and M01640 after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method A)

Male Animal M01638, Urine Pool, 0-24 Hour



Male Animal M01640, Urine Pool, 0-24 Hour

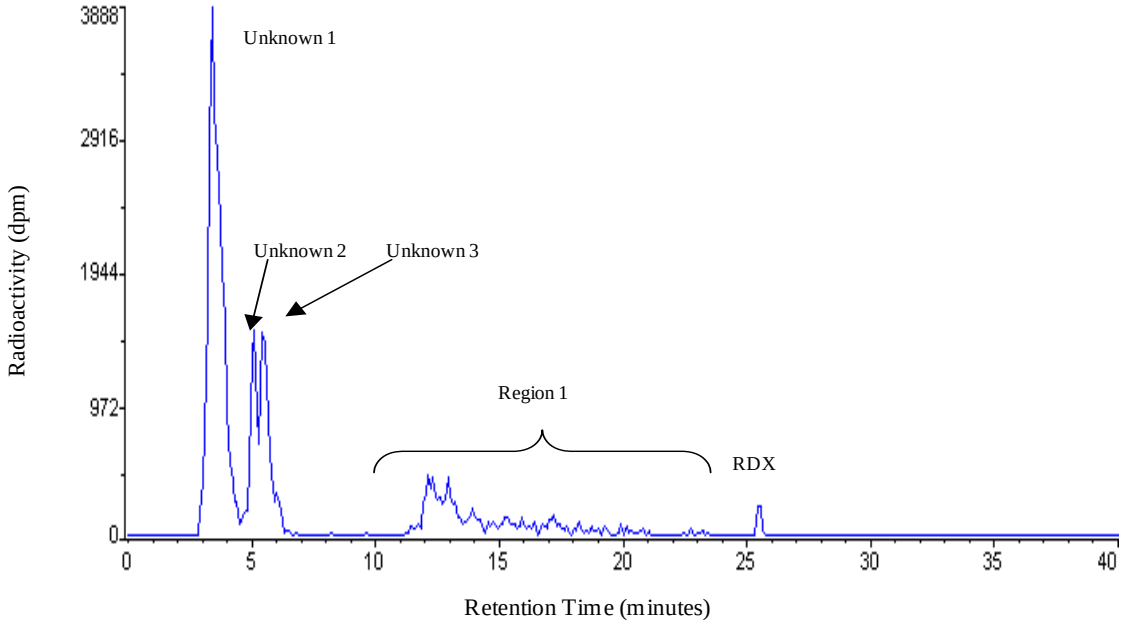
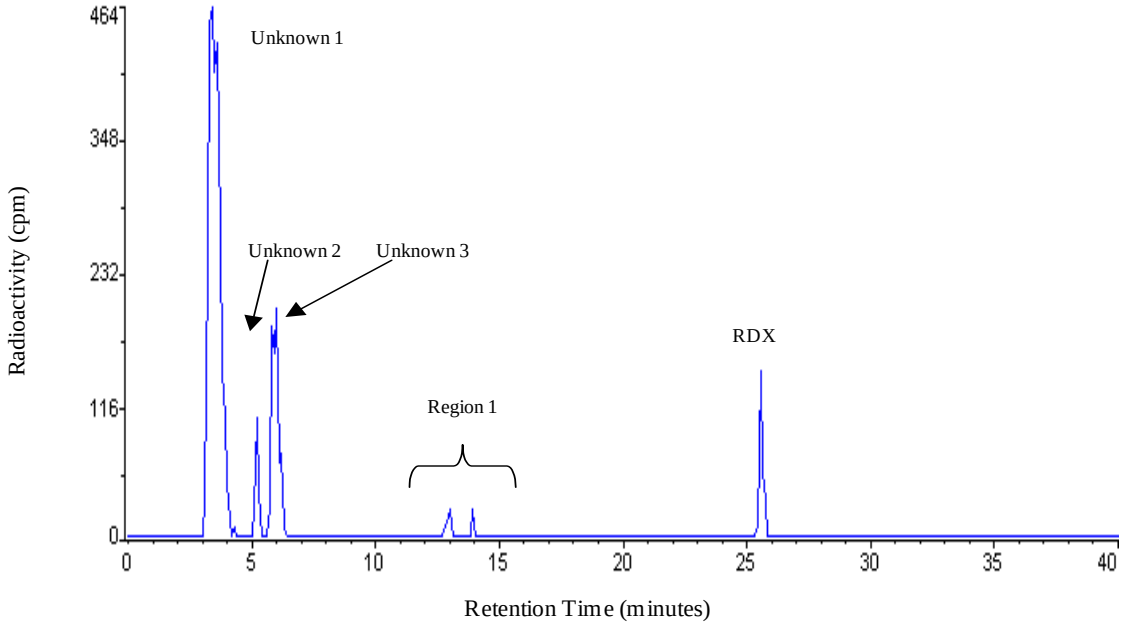


Figure 9
Radiochromatograms of pooled urine (0-24 hours) from female Animals M01643 and M01644 after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method A)

Female Animal M01643, Urine Pool, 0-24 Hour



Female Animal M01644, Urine Pool, 0-24 Hour

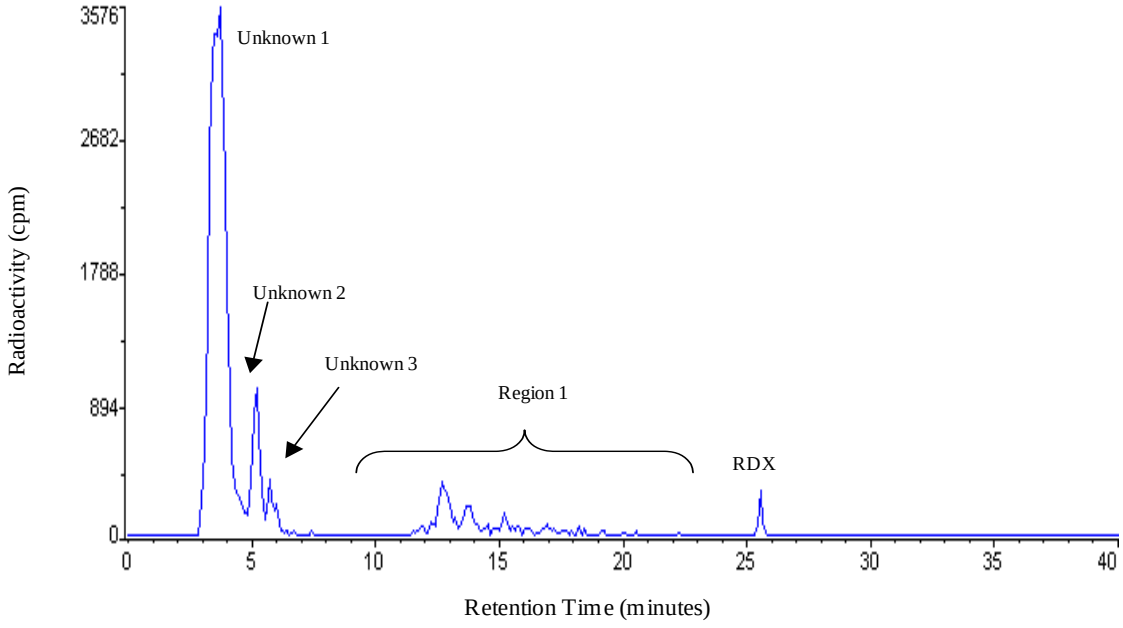
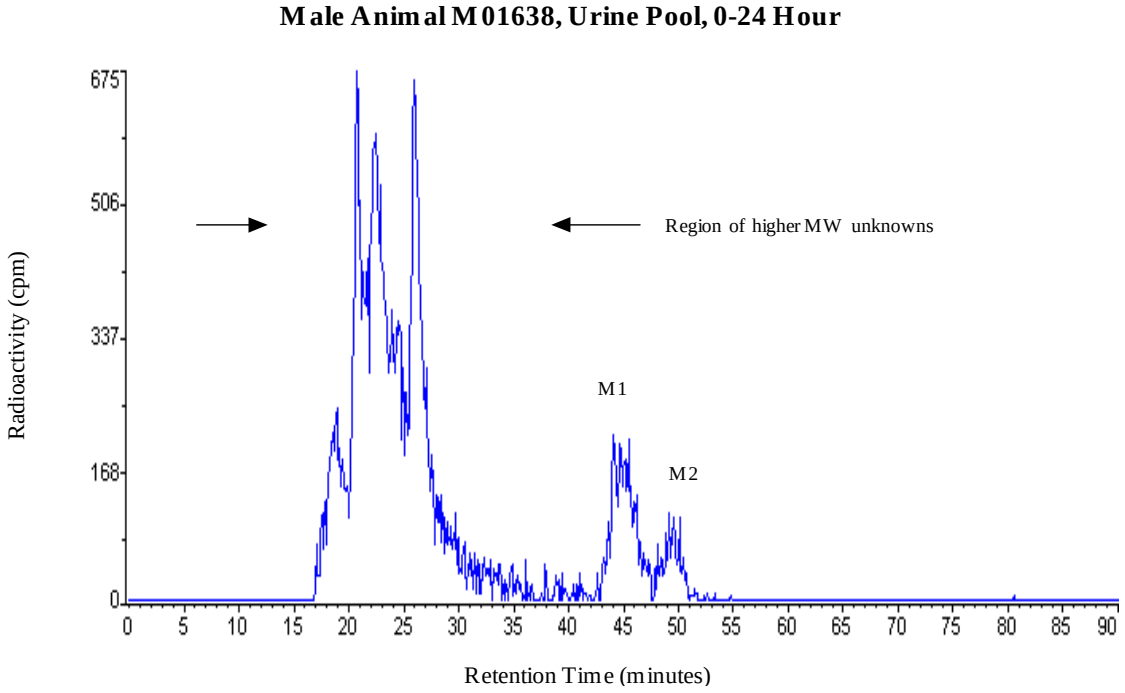
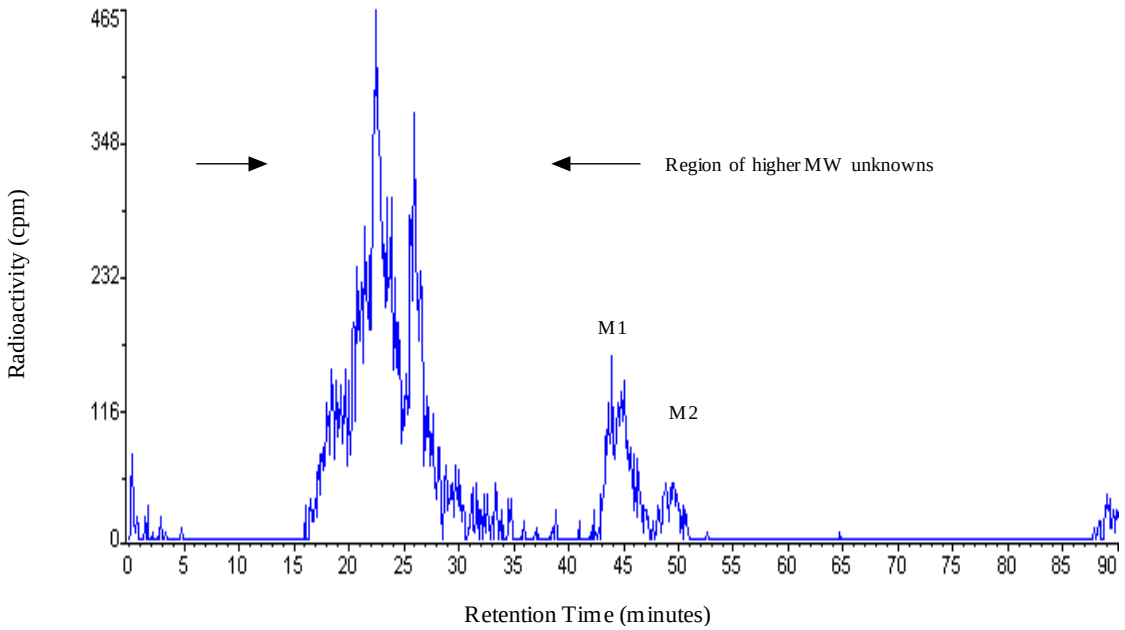


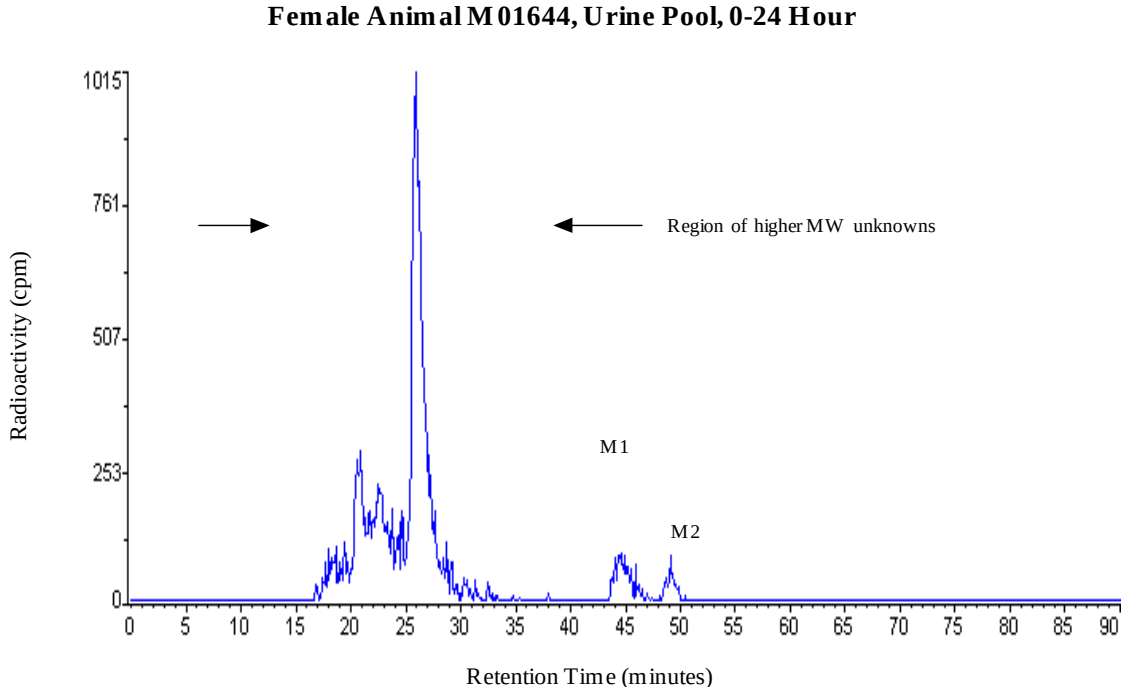
Figure 10
Radiochromatograms of pooled urine (0-24 hours) from male Animals M01638 and M01640 after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method B)





MW Molecular weight.

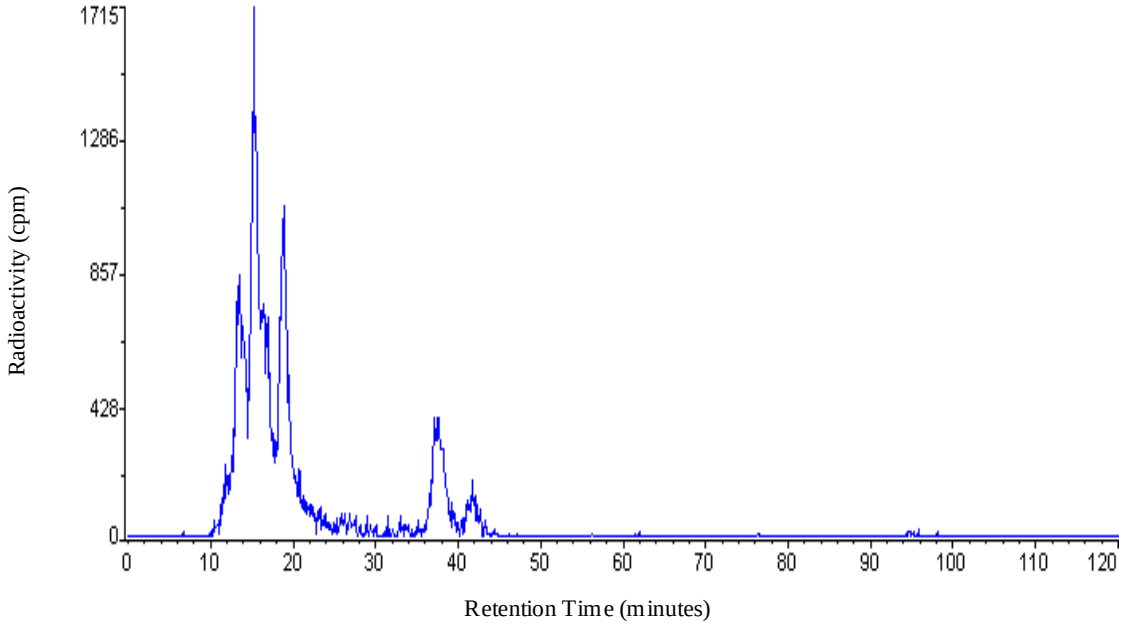
Figure 11
Radiochromatograms of pooled urine (0-24 hours) from female Animal M01644 after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method B)



MW Molecular weight.

Figure 12
Radiochromatograms of pooled urine (0-24 hours) from male Animal M01638 treated with buffer and treated with glucuronidase and sulfataseafter administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method B)

Glucuronide Hydrolysis: 0-24 hour Urine Pool, Male M01638, Treated with Buffer Only



Glucuronide Hydrolysis: 0-24 hour Urine Pool, Male M01638, Treated with Enzyme

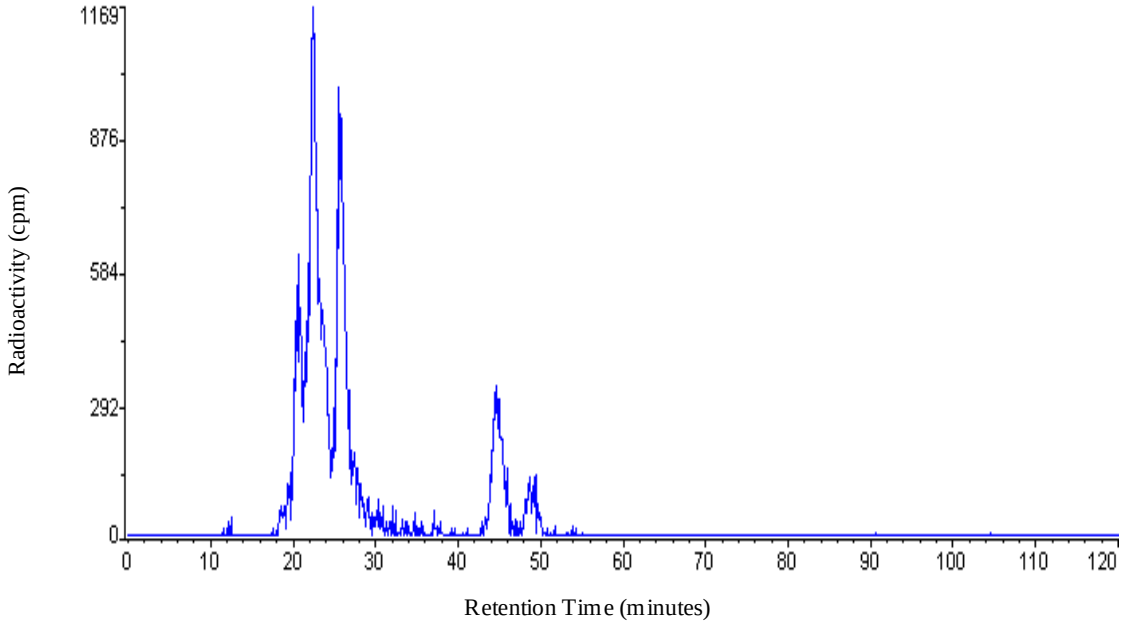


Figure 13
UV chromatograms of RDX, MNX, DNX, and TNX reference substances for plasma analysis (Method A)

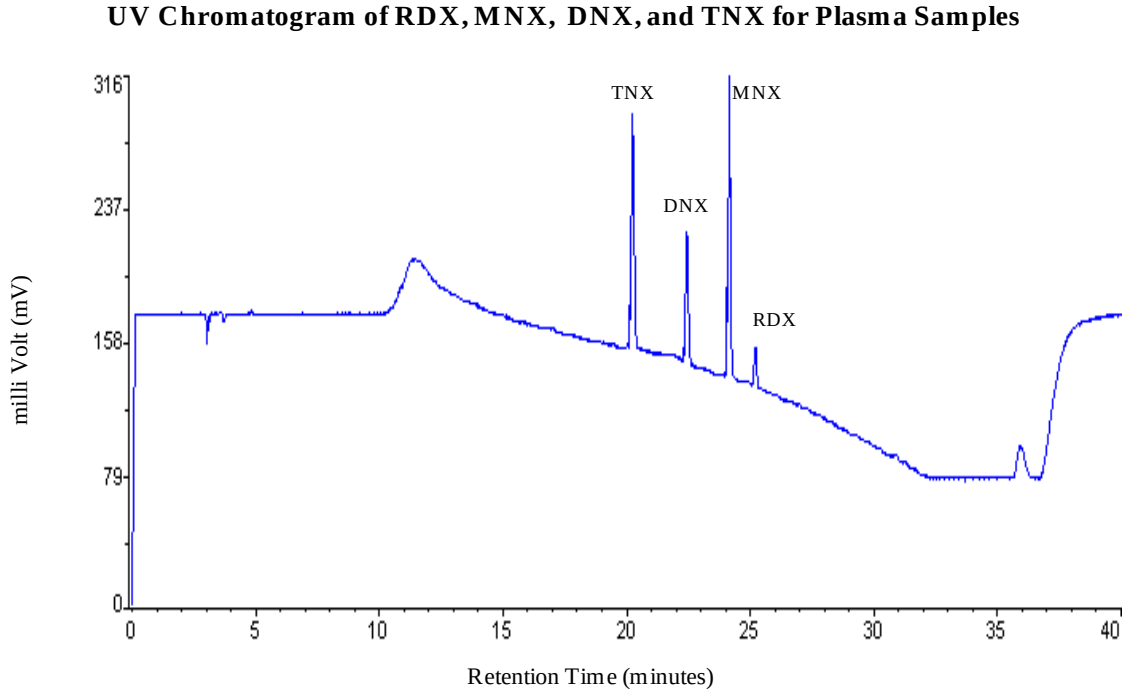


Figure 14
Radiochromatograms of 1- and 6-hour plasma from male Animal M01638 after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method A)

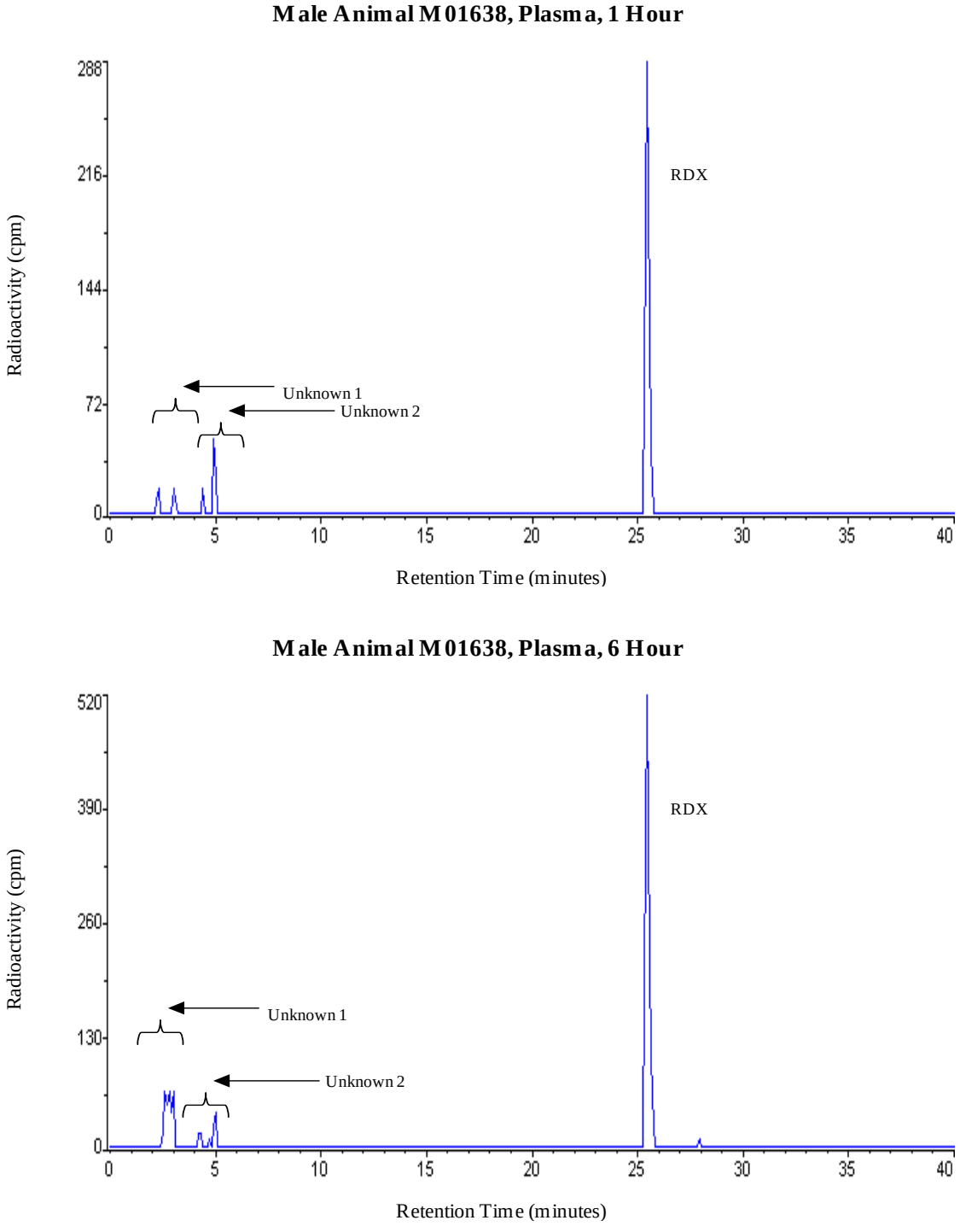
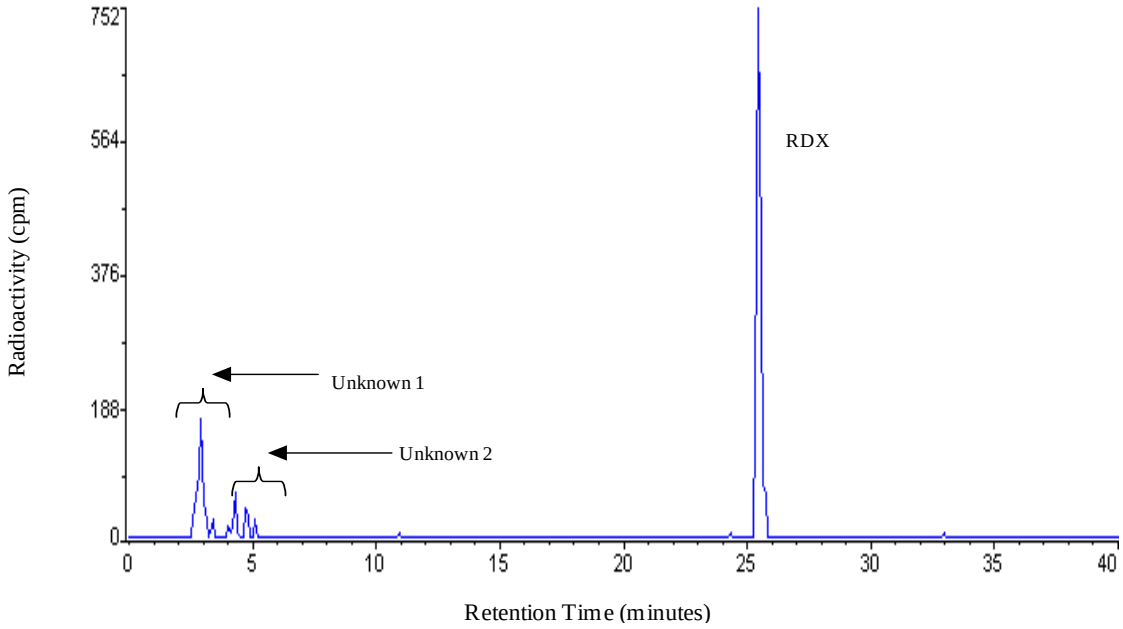


Figure 15
Radiochromatograms of 12- and 24-hour plasma from male Animal M01638 after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method A)

Male Animal M01638, Plasma, 12 Hour



Male Animal M01638, Plasma, 24 Hour

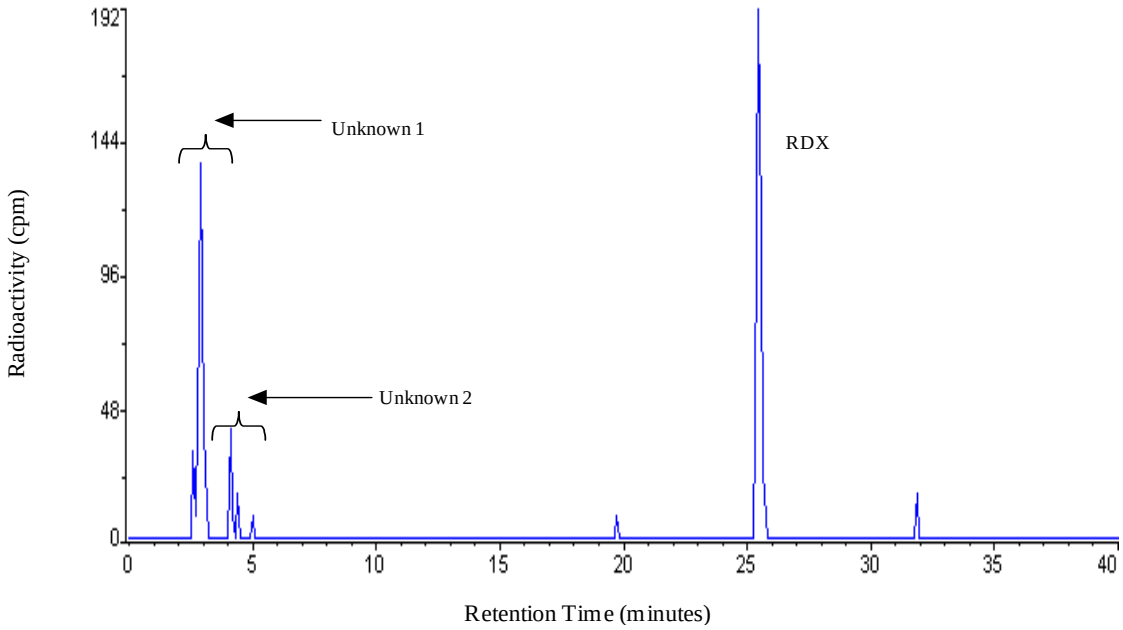
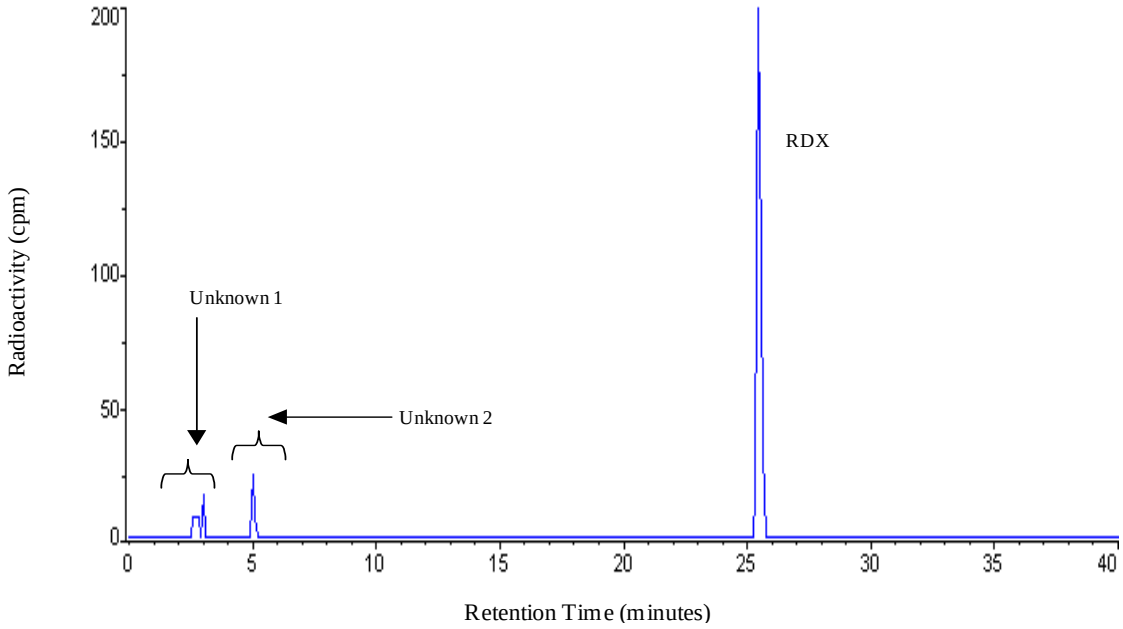


Figure 16
Radiochromatograms of 1- and 6-hour plasma from female Animal M01644 after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method A)

Female Animal M01644, Plasma, 1 Hour



Female Animal M01644, Plasma, 6 Hour

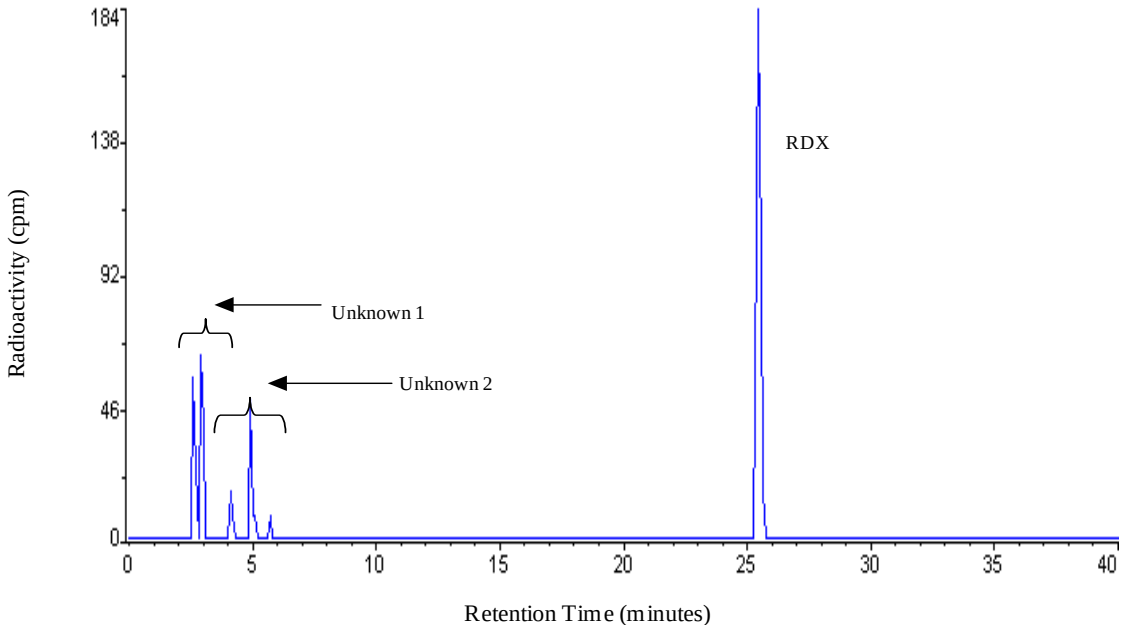
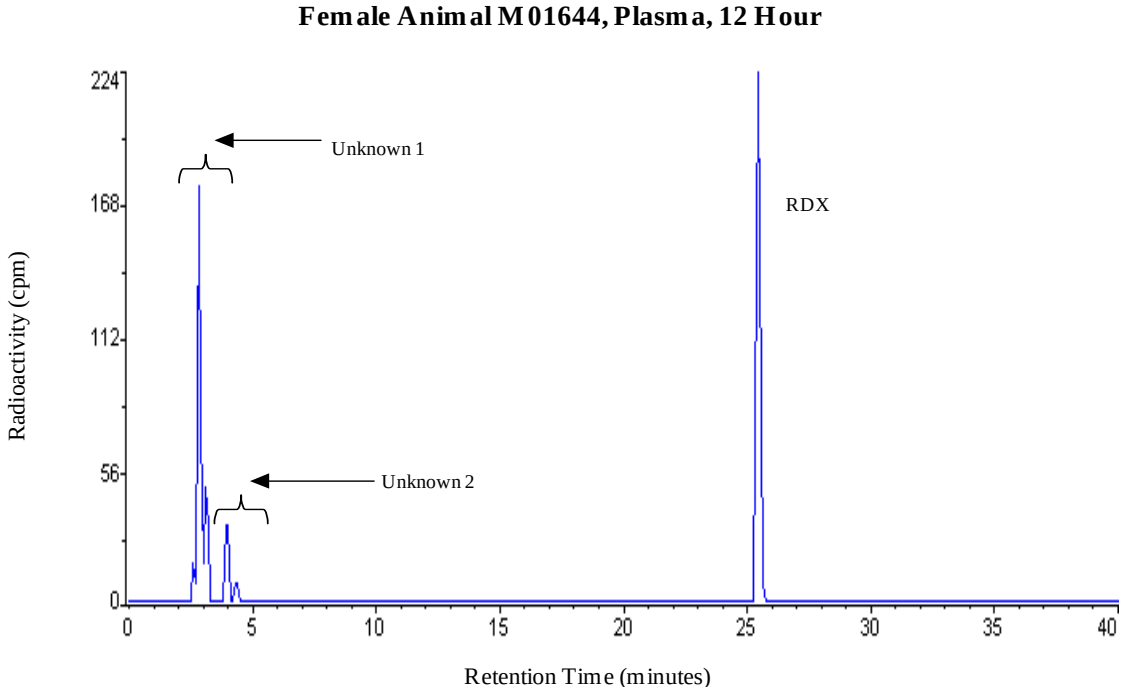


Figure 17
Radiochromatograms of 12- and 24-hour plasma from female Animal M01644 after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method A



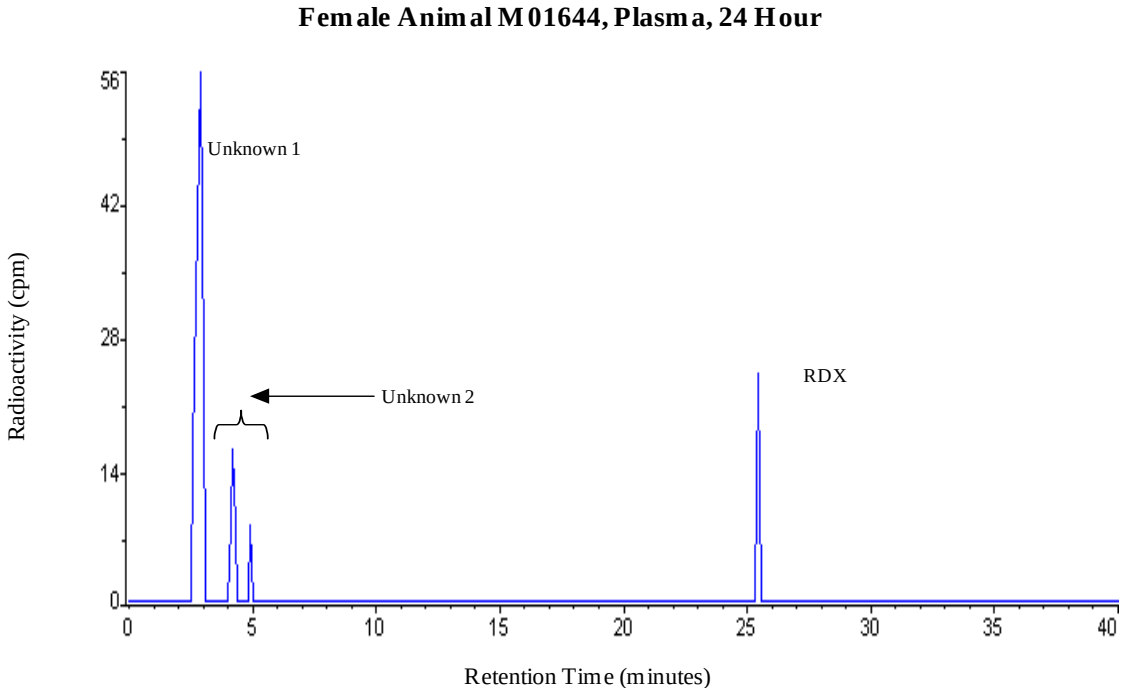
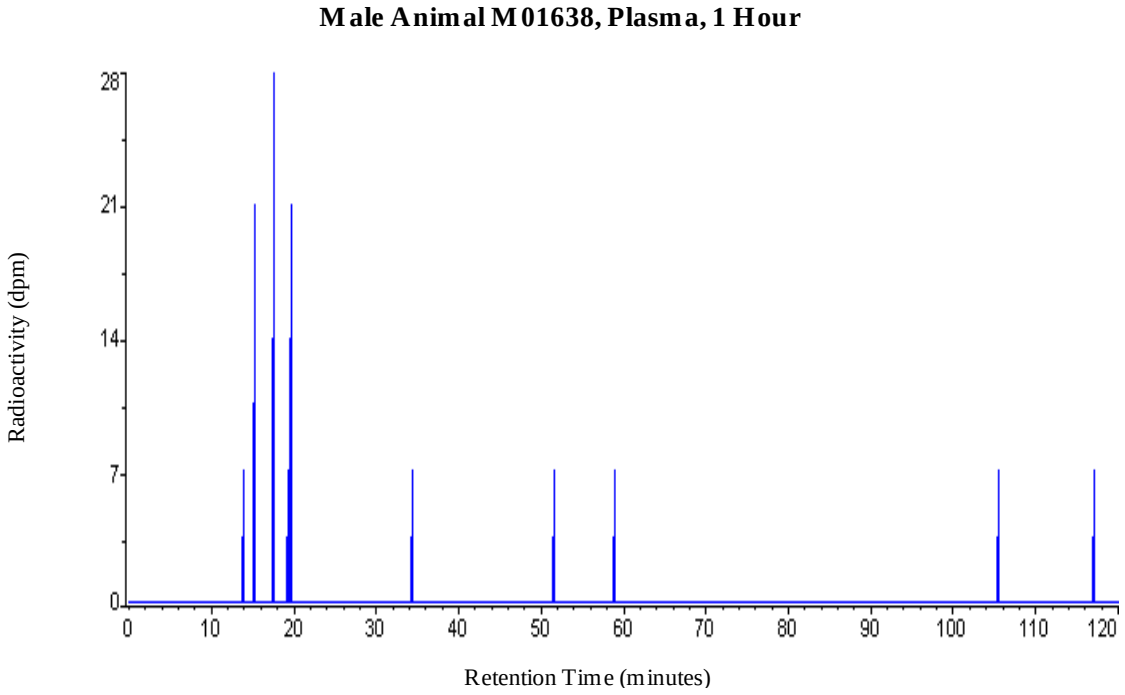


Figure 18
Radiochromatograms of 1- and 6-hour plasma from male Animal M01638 after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method B)



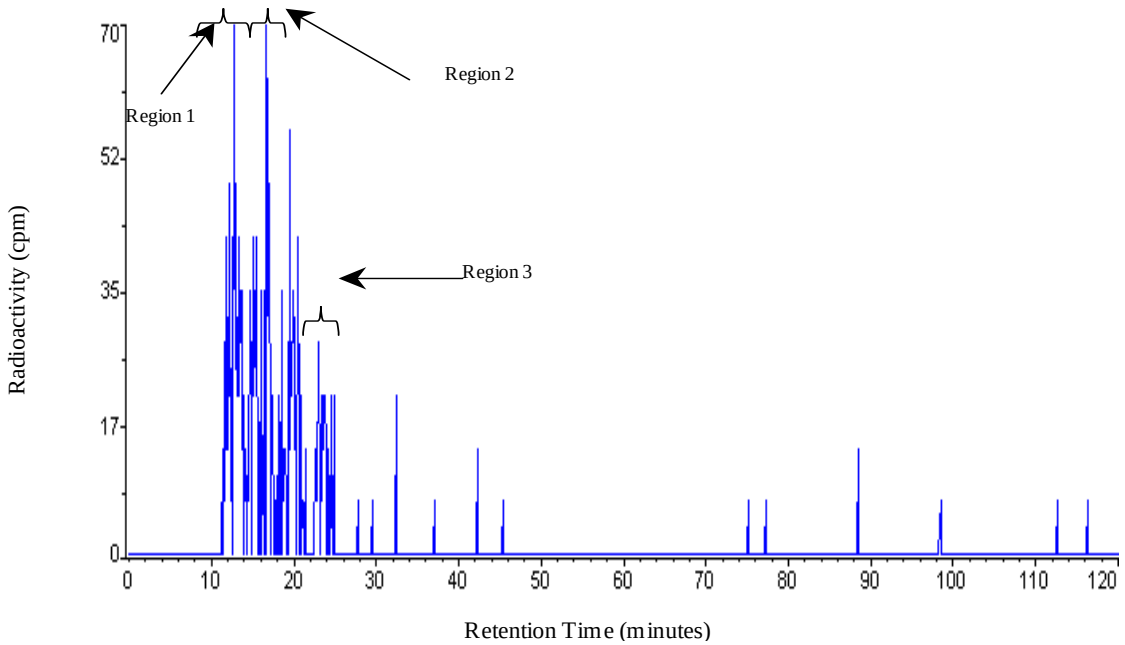
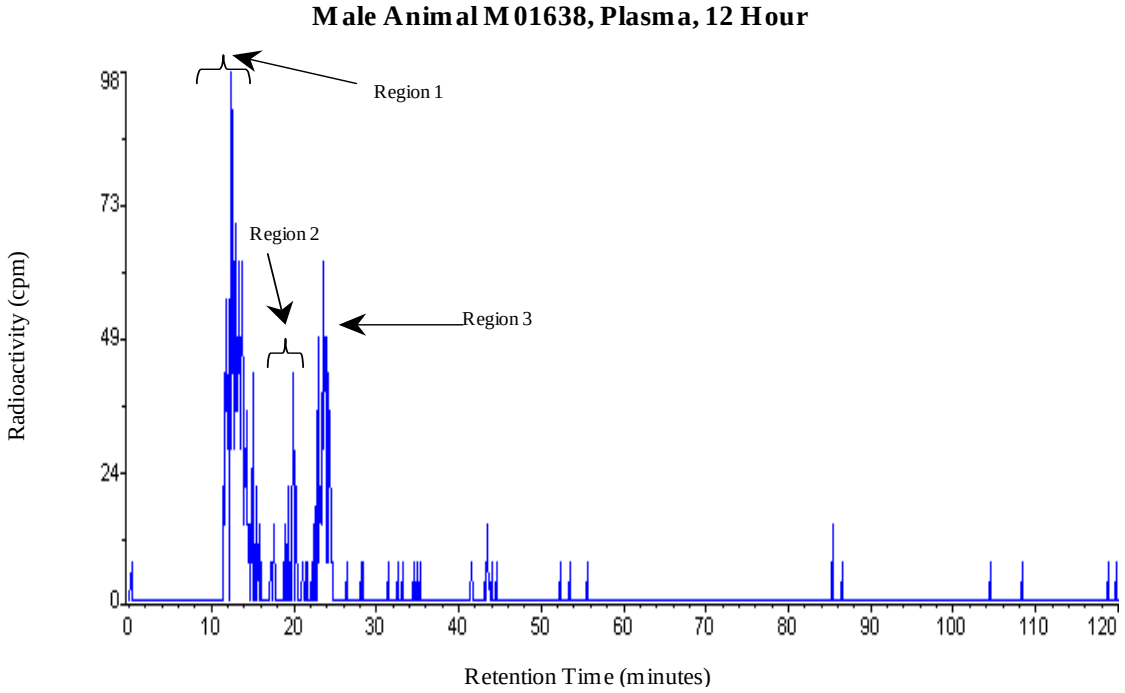


Figure 19
Radiochromatograms of 12- and 24-hour plasma from male Animal M01638 after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method B)



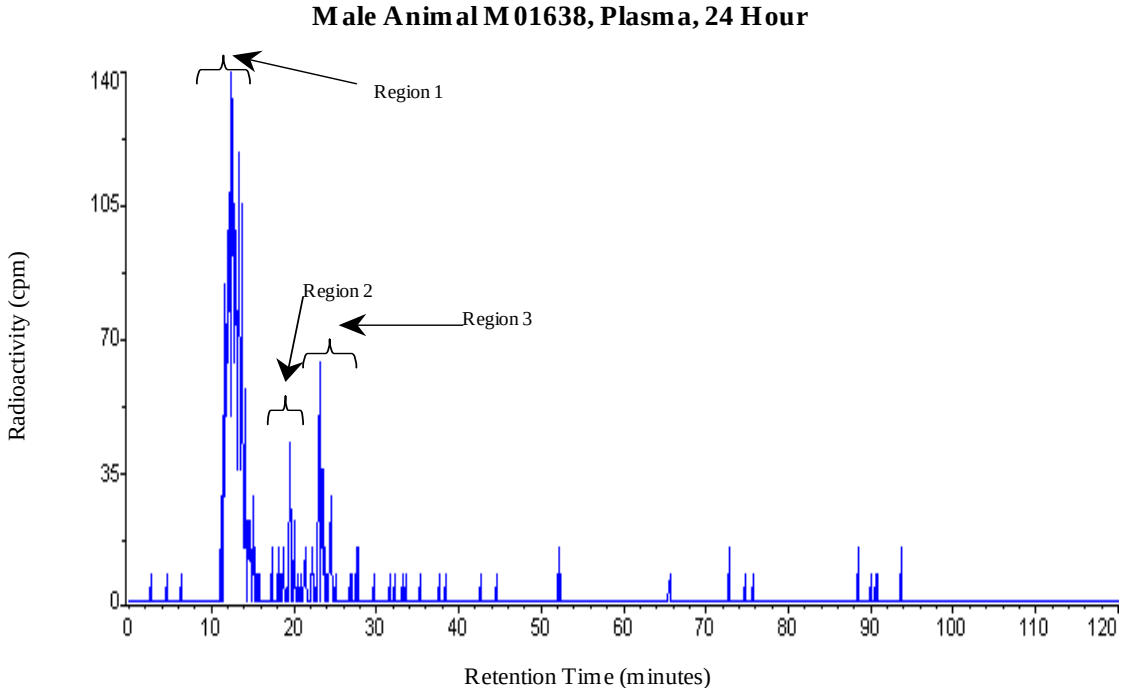


Figure 20
Radiochromatograms of 1- and 6-hour plasma from female Animal M01644 after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method B)

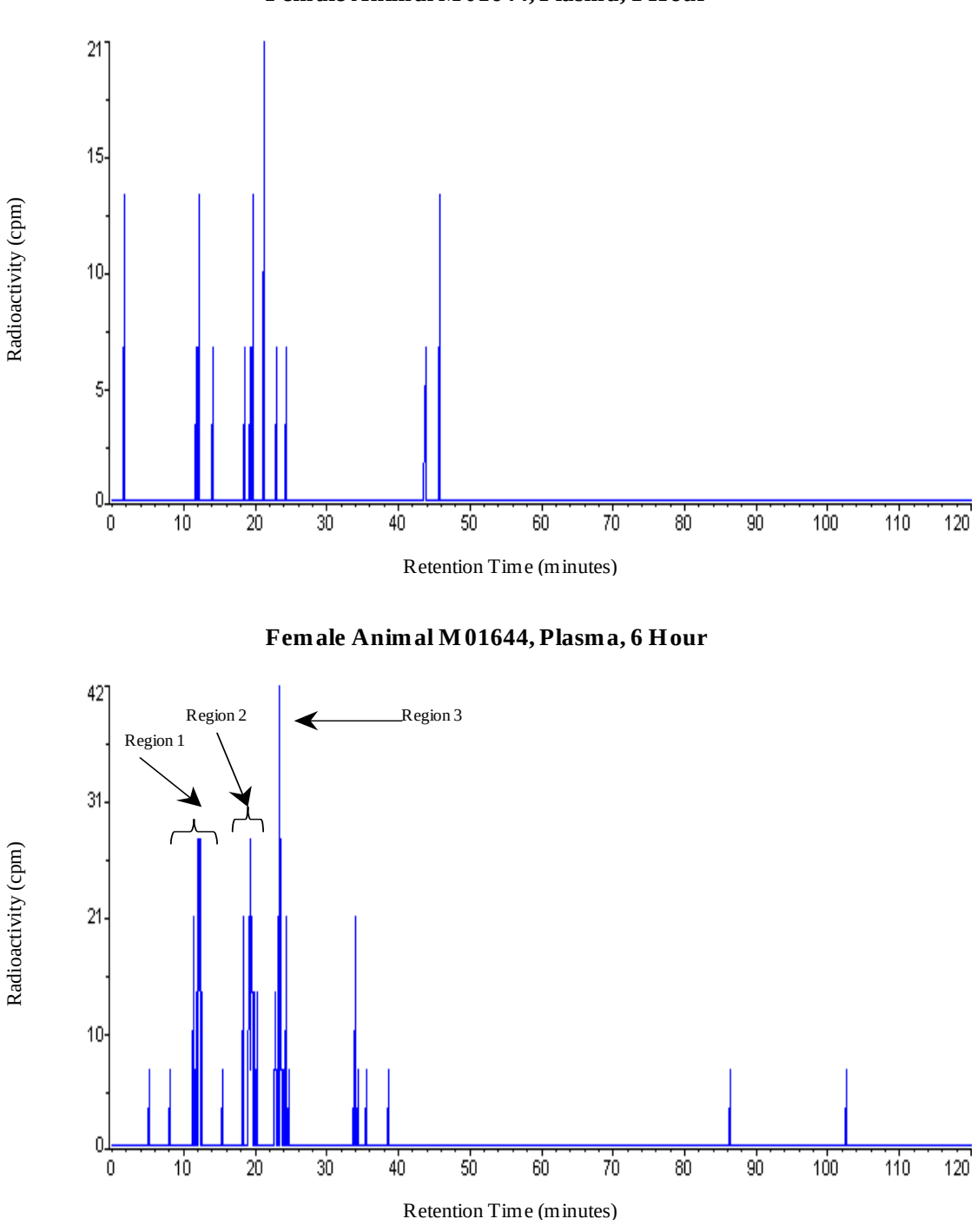
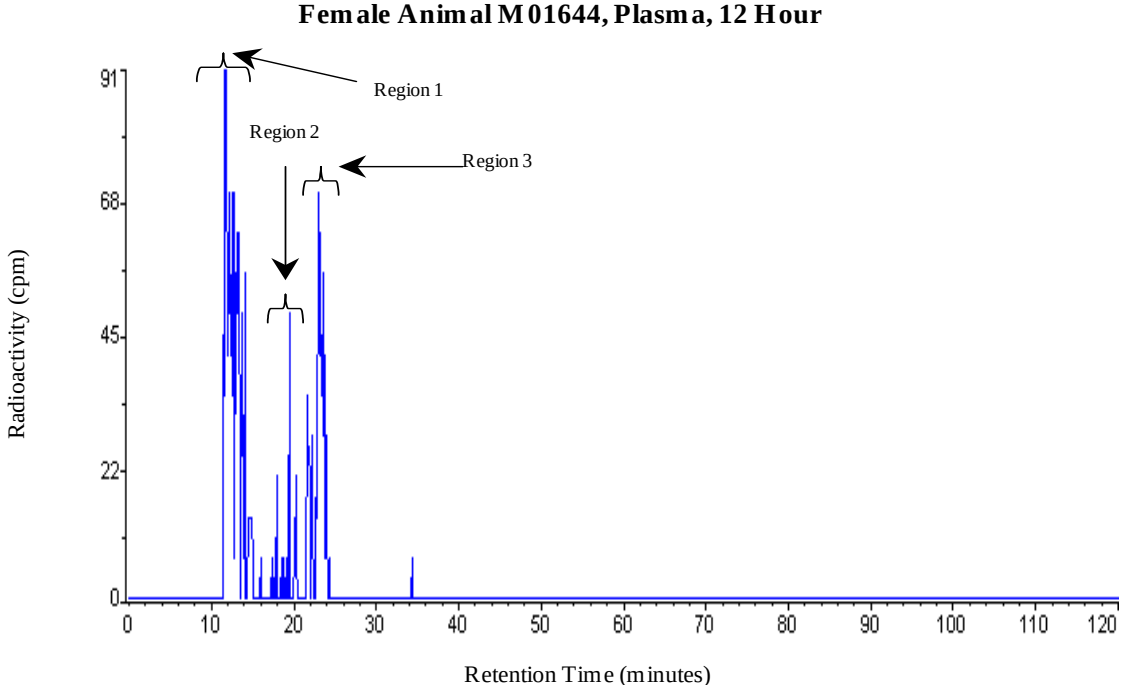


Figure 21
Radiochromatograms of 12- and 24-hour plasma from female Animal M01644 after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method B)



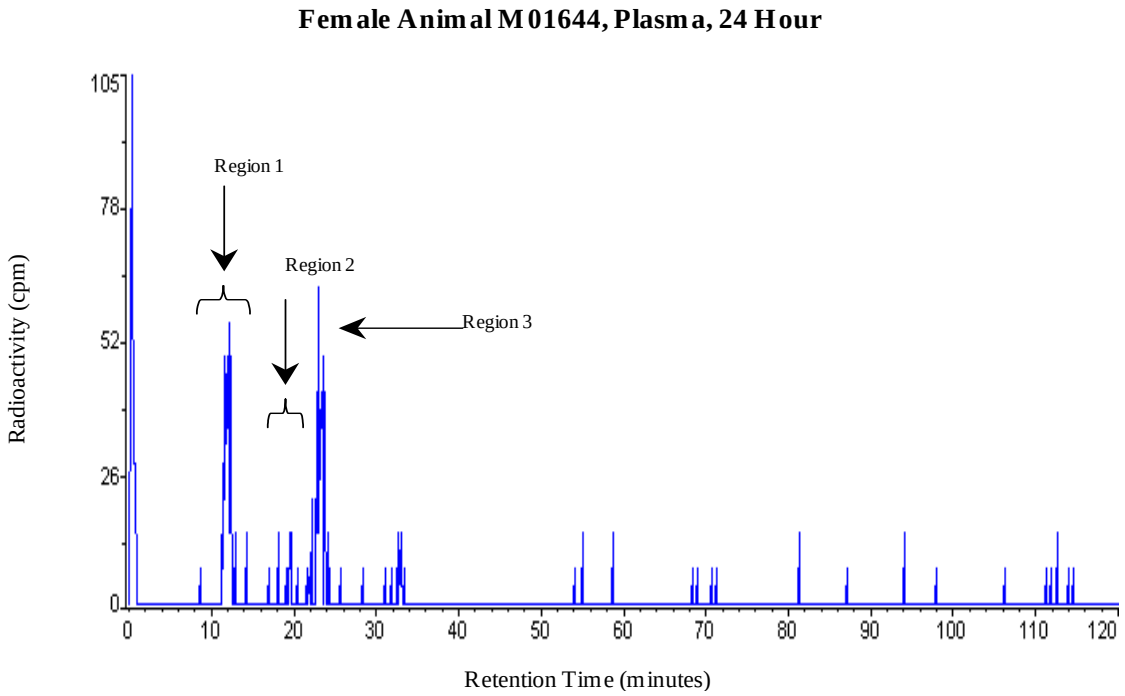


Figure 22
UV chromatogram of RDX, MNX, DNX, and TNX reference substances for liver analysis (Method A)

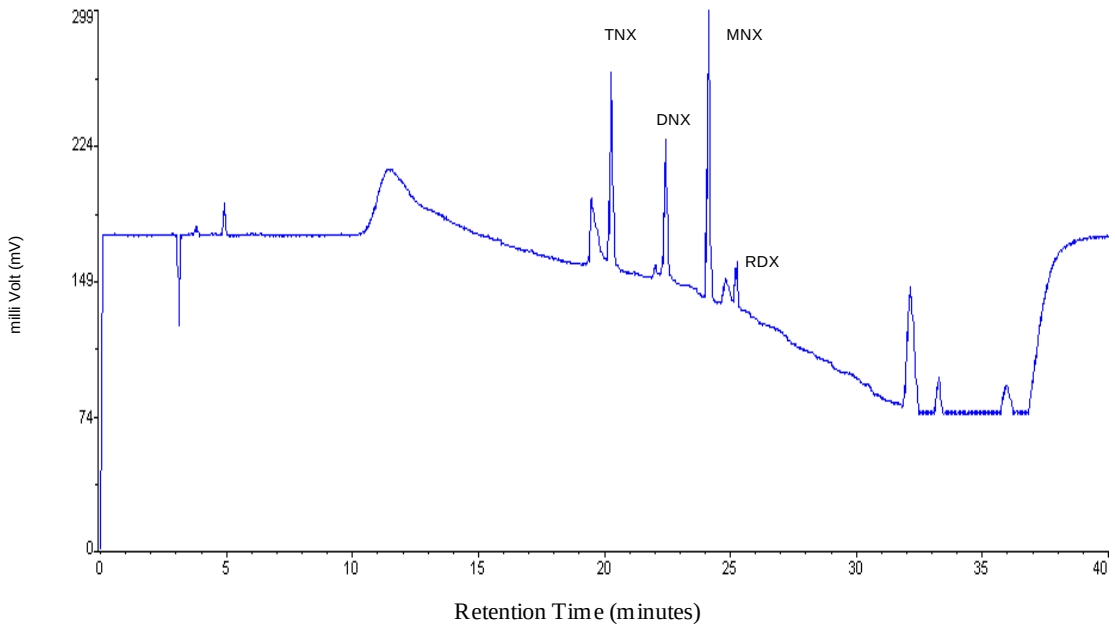
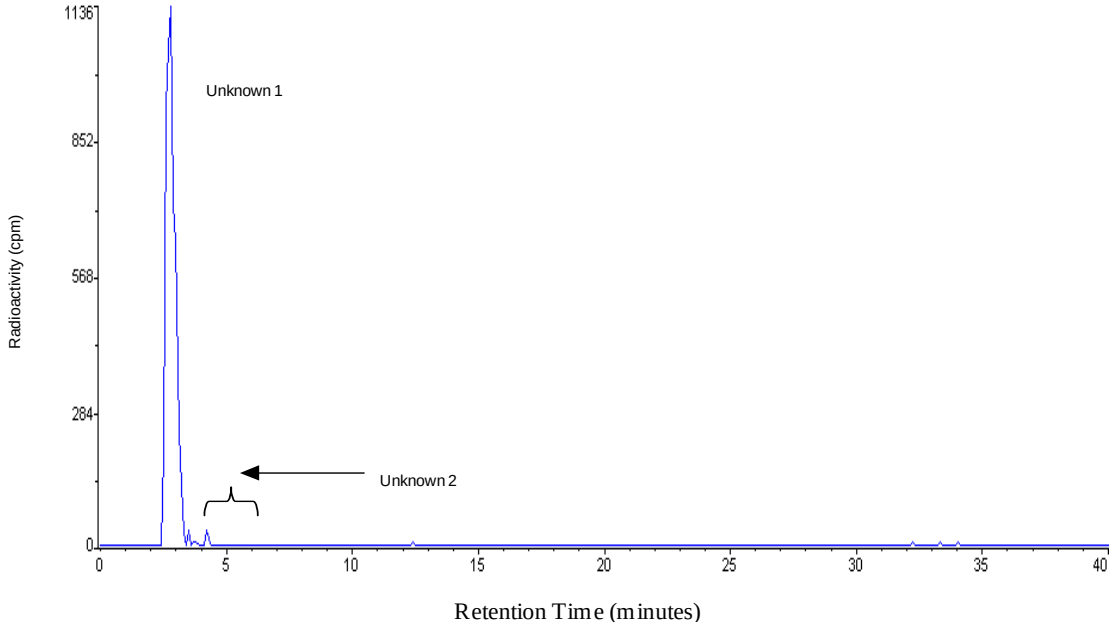


Figure 23
Radiochromatograms of liver (24 hours postdose) from male Animal M01640 and and female Animal M01644 after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method A)

Male Animal M01640, Liver, 24 Hour



Female Animal M01644, Liver, 24 Hour

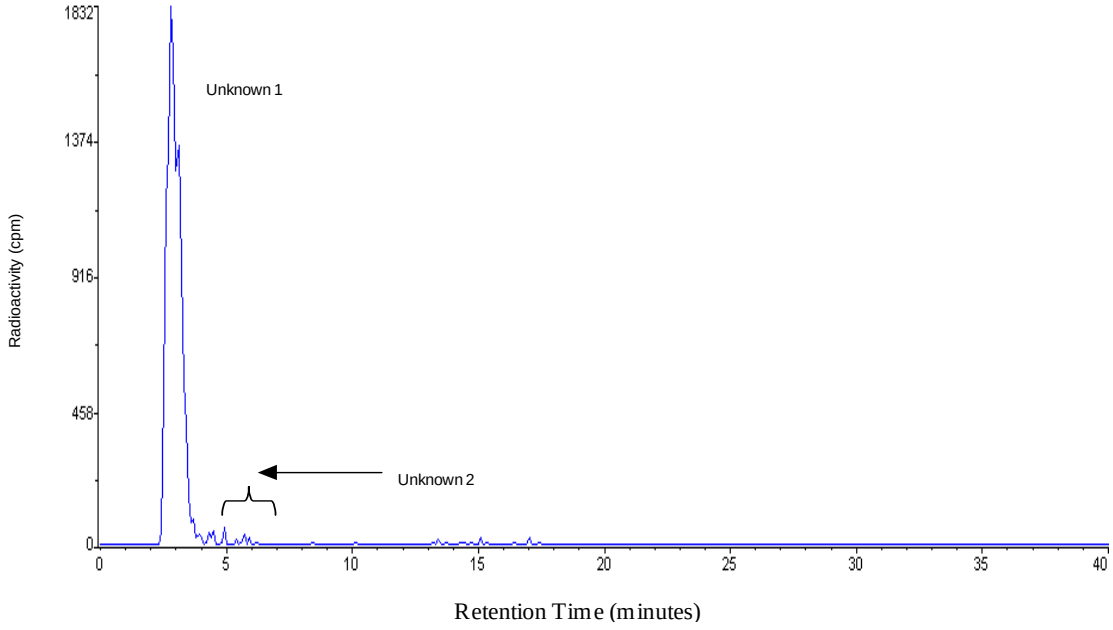
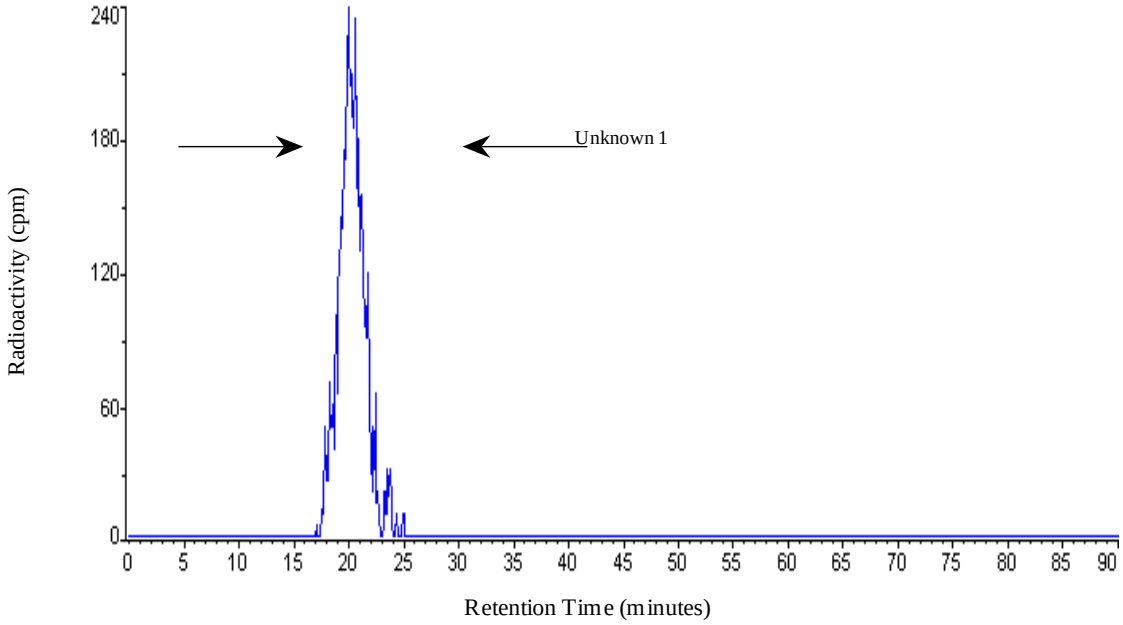


Figure 24
Representative radiochromatograms of liver (24 hours postdose) from male Animal M01640 and female Animal M01644 after administration of a single oral dose of ^{14}C-RDX (45 mg/kg) (Method B)

Male Animal M01640, Liver, 24 Hour



Female Animal M01644, Liver, 24 Hour

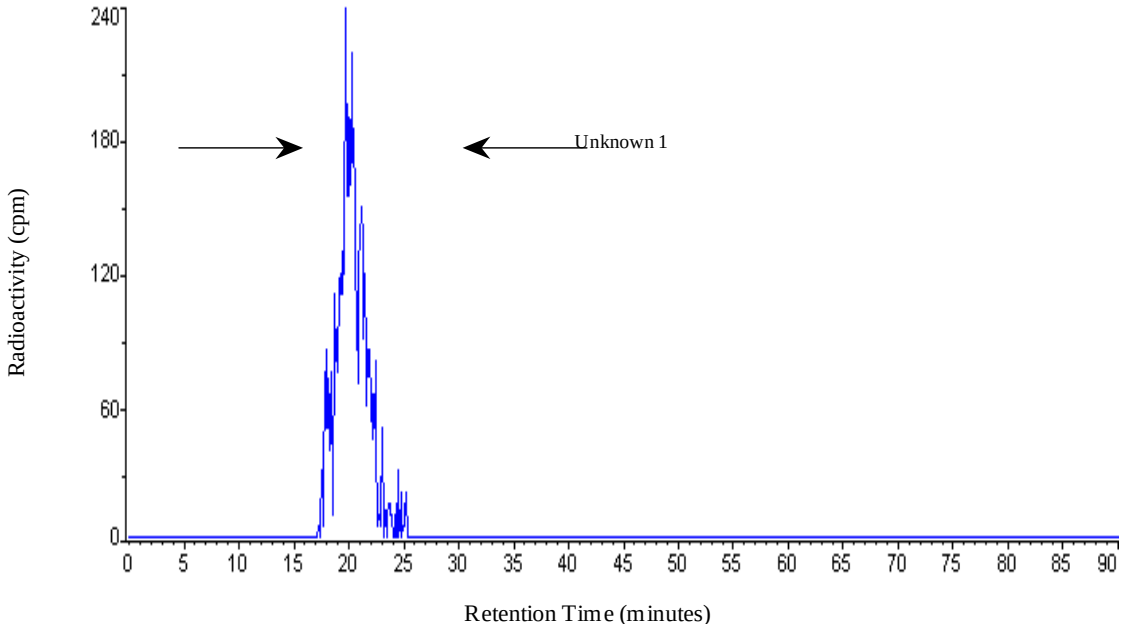
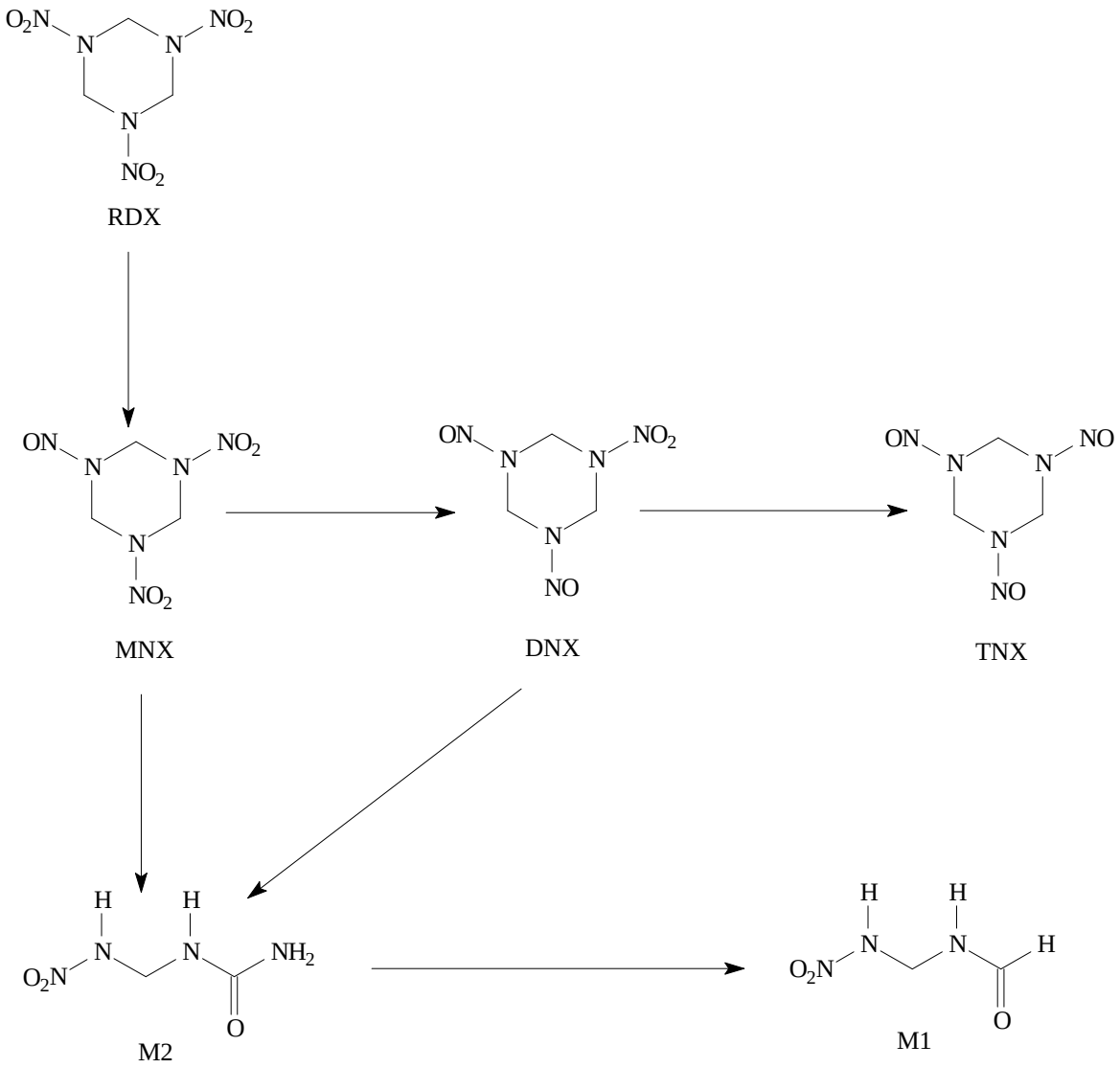


Figure 25
Proposed metabolic pathway for ^{14}C-RDX in male and female minipigs



SIGNATURES

Timothy J. Musick, PhD
Study Director
Drug Metabolism
Covance Laboratories Inc.

02 August 2010
Date

Karen R. Tilch
Study Coordinator
Drug Metabolism
Covance Laboratories Inc.

02 August 2010
Date

Frederic W. Thalacker, PhD
Director
Drug Metabolism
Covance Laboratories Inc.

02 August 2010
Date

APPENDICES

Appendix 1
Protocol, Protocol Amendment, and Protocol Deviations

Final Protocol

Study Title	Absorption, Distribution, Metabolism, and Excretion of ^{14}C-RDX Following Oral Administration to Minipigs
Data Requirement	40 CFR 160, Guideline OPPTS 870.7485
Sponsor	U.S. Army Center for Health Promotion and Preventive Medicine Building E2100 Aberdeen Proving Ground, MD 21010
Study Monitor	Gunda Reddy, PhD, DABT U.S. Army Center for Health Promotion and Preventive Medicine 5158 Blackhawk Road Aberdeen Proving Ground, MD 21010-5483 Telephone: 410.436.7701 Facsimile: 410.436.8258 e-mail: gunda.reddy@apg.amedd.army.mil
Study Director and Study Location	Milan A. Berge, PhD Covance Laboratories Inc. 3301 Kinsman Boulevard Madison, WI 53704-2595 Telephone: 608.242.2667 Facsimile: 608.241.7412 e-mail: milan.berge@covance.com
Covance Study Identification	Covance 7273-121 CMS 29676E
Protocol Issue Date Sponsor Approval Date	Final, 13 July 2004 13 July 2004
Page Number	1 of 11

OBJECTIVE

The purpose of this study is to assess the extent of absorption, distribution, metabolism, and excretion of radioactivity following administration of a single dose of ^{14}C-RDX given to pigs by an oral route of administration. Selected samples will be analyzed for metabolite profiles. In addition, this study will provide selected samples for possible shipment to the Sponsor for analysis under a separate protocol.

REGULATORY COMPLIANCE

This study will be conducted in accordance with the Environmental Protection Agency (EPA) Good Laboratory Practice Standards (40 CFR 160), and in compliance with the following testing guidelines: United States Environmental Protection Agency (EPA) FIFRA, 40 CFR 158, Health Effects Test Guidelines (OPPTS 870.7485); the Wisconsin Department of Health and Family Services, Radiation Protection Section (License No. 48-11805-02); and the applicable Covance Laboratories Inc. (Covance) standard operating procedures.

All procedures in the protocol are in compliance with the Animal Welfare Act Regulations (9 CFR 3).

The protocol, study conduct, data, and final report will be audited by the Quality Assurance Unit (QAU) of Covance in accordance with Covance SOPs. Analyses conducted outside of Covance are the responsibility of the Sponsor and will not be considered within the scope of this study.

This study does not unnecessarily duplicate any previous work.

Major Computer Systems

The major computer systems to be used on this study may include, but not be limited to, the following:

System	Function
Path Tox (PTS)	Direct on-line capture of in-life toxicology data.
Randomization and Data Extension System (RADES)	Used in conjunction with PTS to randomize animals for assignment to treatment groups
Debra	An automated and validated data capture and management system for data collection from balances and scintillation counters for studies using radiolabeled test articles
Metasys	Monitors environmental conditions in the animal facility
Rees	Monitors environmental conditions in storage units
HP Chemstation and Radiomatic Flo-One	Used to capture data from liquid chromatography systems
Softmax Pro	Capture of spectrophotometer data
MassLynx and Analyst	Capture of liquid chromatography/mass spectrometry data

Proposed Study Timetable

Experimental Start Date:	21 July 2004
In-life Initiation:	21 July 2004
In-life Termination:	22 July 2004
Experimental End Date:	21 October 2004

TEST ARTICLES

Radiolabeled Test Article

Test article: ^{14}C-RDX Storage conditions: In a freezer set to maintain –10 to –30°C

Nonradiolabeled Test Article

Test article: RDX Storage conditions: Ambient temperature

REFERENCE SUBSTANCES

In addition to the test articles, the following reference substances will be used for this study.

Reference substance: 1,3,5,-trinitroso-1,3,5-triazacyclohexane (TNX) Storage conditions: Ambient temperature, protected from light
Reference substance: 1-nitroso-3,5-dinitro-1,3,5-triazacyclohexane (MNX) Storage conditions: In a freezer set to maintain –60 to -90°C
Reference substance: 1-nitro-3,5-dinitroso-1,3,5-triazacyclohexane (DNX) Storage conditions: In a freezer set to maintain –60 to -90°C
Reference substance: Methylenedinitramine (MEDINA) Storage conditions: Ambient temperature
Reference substance: 4-nitro-2,4-diazabutanal Storage conditions: In a freezer set to maintain –10 to -30°C

Purity

The chemical purity of the test article will be provided by the Sponsor. Radiopurity of the radiolabeled test article will be confirmed at Covance by a high performance liquid chromatography (HPLC) study-specific procedure based on a method provided by the Sponsor.

Stability
Stability of the bulk test article will be the responsibility of the Sponsor. Stability of the test article in the dose formulation will be determined at Covance by analyzing predose and postdose aliquots of the dose formulation for radiopurity.

Reserve Samples
Since the in-life portion of the study is less than four weeks in duration, retention of a sample of the test article as specified in 40 CFR 160.105(d) is not required.

Test Article Disposition
Unused dose formulations will be discarded. Unused test article will be returned to a site designated by the Sponsor or discarded at the Sponsor's request. Appropriate written certification (e.g., a copy of the current Nuclear Regulatory Commission or state license) will be provided by the Sponsor or designated recipient before the return of any radiolabeled test article.

Safety Precautions
Personnel will follow all safety precautions as required by Covance Policies and Procedures in consideration of Sponsor's Material Safety Data Sheet (MSDS) or other relevant safety information provided by the Sponsor.

EXPERIMENTAL DESIGN

Animals
Species
Pig

Strain and Source
Yucatan minipig, Sinclair Research Center, Inc., Columbia, MO

Weight at Dose Administration
Approximately 8 to 16 kg

Age at Dose Administration
Approximately 2 to 4 months

Number and Sex
Two males and two females on test

Identification
Implantable microchip identification device

Husbandry

Housing

During acclimation, animals will be housed in individual stainless steel cages. During the test period, animals will be housed in individual stainless steel metabolism cages designed for the separation and collection of urine and feces.

Food

Approximately 500 g of Certified Diet #7037 (Harlen Teklad) will be provided twice daily. The manufacturer for nutritional components and environmental contaminant routinely analyzes the diet. Results of specified nutrient and contaminant analyses are on file at Covance. Appropriate treats (that do not require analysis) will be offered in accordance with Covance SOPs for environmental/psychological enrichment.

Water

Ad libitum, provided fresh daily. The water source is the city of Madison, WI. Samples of the water are routinely analyzed for specified microorganisms and environmental contaminants. The results are on file at Covance.

Contaminants

There are no known contaminants in the food or water that would interfere with this study.

Environment

Environmental controls for the animal room will be set to maintain a temperature of 16 to 27°C, a relative humidity of 50±20%, and a 12-hour light/12-hour dark cycle. The 12-hour dark cycle may be interrupted to accommodate study procedures.

Acclimation

Approximately 2 weeks

Randomization

Via computer-generated random numbers for assignment to the study based on body weight

Justification

The minipig is a suitable nonrodent species for developing data on the absorption, distribution, metabolism, and excretion of a test chemical to aid in the evaluation of test results from toxicology studies and in the extrapolation of data for environmental safety assessment. The number of animals is the minimum number required to obtain scientifically valid results and to ensure adequate sample size for analysis.

Reason for Dosing Route

Potential exposure is by the oral route.

Study Design

Group Designations and Dose Levels

Group	Number of Animals Male	Number of Animals Female	Target Dose Level (mg/kg)	Dose Route	Target Dose Volume (mL/kg)	Samples Collected
1	2	2	45	Oral	5	Blood, tissues, urine, and feces

Dosing Procedures

Dose Preparation and Analyses

The appropriate quantities of radiolabeled and nonradiolabeled test articles will be combined in 0.5% carboxymethylcellulose (CMC) in water to prepare the dose formulation. Predose and postdose samples of each radiolabeled dose formulation will be taken at the time of dose administration to determine radioactivity concentration and homogeneity by LSC and gravimetric analysis, in accordance with Covance SOPs. Predose and postdose samples will be stored in a freezer set to maintain –10 to -30°C except during preparation for analysis.

Dose Administration

The volume of radiolabeled dose formulation to be administered to each animal will be calculated based on body weights taken on the day of dosing. The actual amount administered will be determined by weighing the dose syringe before and after dose administration.

Animals will not be fasted prior to dose administration.

The oral dose will be administered via oral gavage. Prior to withdrawing the gavage tube, the tube will be flushed with approximately 5 mL of dose vehicle. Each gavage tube will be retained for radioanalysis.

Observation of Animals

Antemortem Observations

Twice daily (a.m. and p.m.), animals will be observed for mortality and signs of pain and distress. Cageside observations for general health and appearance will be done once daily and documented in the study data, in accordance with Covance SOPs.

Body Weights

At arrival, weekly during acclimation, at randomization, and on the day of dose administration.

Sample Collection

The following samples will be collected for radioanalysis.

Excretion

The following samples will be collected for analysis of excretion of radioactivity.

Urine will be collected from all animals at predose (at least 12 hours) and at 0-6, 6-12, and 12-24 hours postdose. Urine will be collected in plastic containers surrounded by dry ice. The weight of each sample will be recorded using the Debra system.

Feces will be collected from all animals at predose (at least 12 hours) and at 0-24 hours postdose. Feces will be collected at room temperature and transferred to plastic containers and stored in a freezer set to maintain –10 to -30°C. The weight of each sample will be recorded using the Debra system.

After the last excreta collections, cages will be washed and wiped with a solution of 1% trisodium phosphate in water and gauze pads. The cage wash samples and gauze will be collected into separate plastic containers and the weight of each cage wash sample will be recorded using the Debra system.

Blood and Plasma
Blood (approximately 10 mL) will be collected via the anterior vena cava into tubes containing sodium heparin anticoagulant from all animals at 1, 6, and 12 hours postdose. Blood samples will be placed on wet ice, in a chilled Kryorack, or stored at in a refrigerator set to maintain 3 to 5°C until aliquoted and centrifuged to obtain plasma, buffy coat, and cellular fraction.

Tissue Distribution
At 24 hours postdose, animals will be sacrificed via exsanguination under sodium pentobarbital anesthesia. Animals may be anesthetized with telazol prior to sacrifice and after blood collections. Prior to sacrifice, blood (approximately 30 mL) will be collected via the anterior vena cava into tubes containing sodium heparin anticoagulant. Blood samples will be placed on wet ice, in a chilled Kryorack, or stored in a refrigerator set to maintain 3 to 5°C until aliquoted and centrifuged to obtain plasma, buffy coat, and cellular fraction.

The following matrices will be collected from each animal, as appropriate:

Brain	Liver	Small intestine (with contents)
Fat (abdominal)	Lungs	Stomach (with contents)
Heart	Muscle (skeletal)	Testes (as applicable)
Kidneys	Ovaries (as applicable)	
Large intestine (with contents)	Skin	

Tissues will be excised, rinsed with saline and blotted dry (as appropriate), weighed, and placed on dry ice. In addition, 5 approximately 2-3 g samples of each liver will be flash frozen in liquid nitrogen for possible shipment to the Sponsor. The residual carcass will be discarded.

Acceptable Time Ranges
The acceptable time ranges for sample collections are summarized in the following table.

Scheduled Collection Time	Acceptable Time Range
0 – 15 minutes	± 1 minute
16 - 30 minutes	± 2 minutes
31 - 45 minutes	± 3 minutes
46 - 60 minutes	± 4 minutes
61 minutes – 2 hours	± 5 minutes
2 hours 1 minute – 8 hours	± 10 minutes
> 8 hours – 24 hours	± 20 minutes
> 24 hours	± 60 minutes

Unscheduled Sacrifices and Deaths
At the discretion of the study director or laboratory animal veterinarian, animals will be sacrificed via exsanguination under sodium pentobarbital anesthesia or overdose of Beuthanasia D based on the general health of the animals. Animals may be necropsied and/or examined macroscopically. Selected samples listed above may be collected for analysis.

Sample Identification and Storage
Samples will be identified with the Covance study number, sex, radioisotope, animal number, sample matrix, sample number, and collection time or interval.

All samples, except blood, will be stored in a freezer set to maintain –10 to -30°C before and after analysis. Blood will be stored on wet ice, in a chilled Kryorack, or in a refrigerator set to maintain 3 to 5°C until aliquots are taken for radioanalysis. Plasma will be harvested by centrifugation and stored in a freezer set to maintain –10 to -30°C. The cellular fraction of the blood and the buffy coat will be saved for possible analysis.

Sample Preparation and Radioanalysis
Samples will be analyzed for content of radioactivity by liquid scintillation counting (LSC) according to Covance SOPs. Each sample will be homogenized before radioanalysis. All samples will be analyzed in duplicate if sample size allows and counted for at least 5 minutes or 100,000 counts.

Characterization of Metabolites
Selected samples of urine, feces, plasma, and tissues will be analyzed for parent compound and metabolites. Selected samples may be analyzed by high performance liquid chromatography (HPLC) for parent compound and any metabolites. After review of the data, samples may be pooled by sex and collection time or interval. In addition, selected samples may be analyzed by liquid chromatography/mass spectrometry (LC/MS) for metabolite identification.

Statistical Analyses
Statistical analyses will include such parameters as mean and standard deviation, as appropriate.

REPORT

One copy of the draft report will be submitted to the Sponsor for review and comment. A final report will then be provided to the Sponsor. The report will include, but will not be limited to, the following items.

Experimental Design and Methods
As defined by the protocol, protocol amendments, and any protocol deviations.

Data and Results
Concentration and homogeneity of radioactivity in the dose preparation
Amount of each dose administered in mg/kg of body weight, mg/animal, µCi/kg of body weight, and µCi/animal
Specific activity of the test article
Concentrations of radioactivity in blood, plasma, and tissues
Tissue:plasma concentration ratios
Amounts of radioactivity in tissues (tissues collected in their entirety) and excreta, expressed as percent of the total administered dose
Graphs of selected excretion patterns
Results of metabolite profiling and sample characterization

If comments on the draft report are not received within 6 months after submission, it will be assumed that the Sponsor authorizes finalization. Any subsequent changes after the report is finalized will be included in a report amendment, with possible additional charges for preparation of the amendment.

MAINTENANCE OF DATA RECORDS AND SPECIMENS

Specimens and the following records to be maintained and transferred to the archives of Covance will include, but will not be limited to:

Protocol and any amendments
Dose preparation
Dose analysis
Animal receipt
Acclimation
Body weights
Randomization
Dose administration
Sample collection
Antemortem observations
Radioanalysis procedures
Sample weights

LSC printouts
Laboratory notebooks
High-performance liquid chromatography and/or thin-layer chromatography data, as appropriate
Study correspondence
Statistical records
Final report

The following supporting records to be retained at Covance but not archived with the study data will include, but will not be limited to:

Feed analysis records
Water analysis records
Animal room temperature and humidity records
Refrigerator/freezer temperature records
Instrument calibration and maintenance records
United States Department of Agriculture records

The original signed protocol, the original signed report, the study correspondence, and all raw data captured on durable media will be permanently archived in the storage facilities of the Covance site at which the work was performed. All paper data and specimens will be retained at Covance for at least 10 years, after which time the Sponsor will be contacted to determine whether these articles should be returned or retained. The Sponsor will be advised of the financial implications of each option at that time.

PROTOCOL APPROVAL

Gunda Reddy, PhD, DABT
Study Monitor
U.S. Army

16 July 2004
Date

Milan A. Berge, PhD
Study Director
Drug Metabolism
Covance Laboratories Inc.

13 July 2004
Date

Jon F. Denissen, PhD
Executive Director
Drug Metabolism
Covance Laboratories Inc.

13 July 2004
Date

COVANCE
THE DEVELOPMENT SERVICES COMPANY

PROTOCOL AMENDMENT NO. 1

Covance 7273-121

Absorption, Distribution, Metabolism, and Excretion of ^{14}C-RDX Following Oral Administration to Minipigs

Sponsor:	U.S. Army Center for Health Promotion and Preventive Medicine, Aberdeen Proving Ground, MD
Study Monitor:	Gunda Reddy, PhD, DABT
Study Location:	Covance Laboratories Inc., Madison, WI
Study Director:	Milan A. Berge, PhD

This amendment modifies the following portions of the protocol:

Effective 13 July 2004

1. **Page 4, Reserve Samples.** To indicate that reserve samples are required, replace this section with the following:

   Since the experimental portion of the study is more than four weeks in duration, the Sponsor will assume responsibility for retention of a sample of the test articles and reference substances in accordance with the applicable regulations.

2. **Page 7, Blood and Plasma.** To correct a typographical error, in Sentence 2, replace "3 to 5°C" with "2 to 8°C."

3. **Page 7, Tissue Distribution.** To correct a typographical error, in Paragraph 1, Sentence 4, replace "3 to 5°C" with "2 to 8°C."

4. **Page 8, Sample Identification and Storage.** To correct a typographical error, in Paragraph 2, Sentence 2, replace "3 to 5°C" with "2 to 8°C."

Effective 14 July 2004

5. **Page 7, Tissue Distribution.** To indicate that gastrointestinal tract tissues and contents will be collected separately, replace "Large intestine (with contents)" with "Large intestine" and "Large intestine contents and wash."

6. **Page 7, Tissue Distribution.** To indicate that gastrointestinal tract tissues and contents will be collected separately, replace "Small intestine (with contents)" with "Small intestine" and "Small intestine contents and wash."

7. **Page 7, Tissue Distribution.** To indicate that gastrointestinal tract tissues and contents will be collected separately, replace "Stomach (with contents)" with "Stomach" and "Stomach contents and wash."

Effective 21 July 2004

8. **Page 3, Proposed Study Timetable.** To reflect the updated dates due to a delay in dosing, replace this section with the following:

Experimental Start Date:	16 August 2004
In-life Initiation:	16 August 2004
In-life Termination:	17 August 2004
Experimental End Date:	21 January 2005

Effective 30 July 2004

9. **Page 4, Weight at Dose Administration.** To address the additional weight gain for the animals due to a delay in the dosing, replace this section with the following:

   Approximately 10 to 18 kg

10. **Page 4, Age at Dose Administration.** To update the age of the animals at dose administration due to a delay in the dosing, replace "4 months" with "5 months."

11. **Page 5, Acclimation.** To address the longer acclimation due to a delay in the dosing, replace this section with the following:

    At least 2 weeks

12. **Page 6, Group Designation and Dose Levels.** To include the amount of radioactivity each animal will receive, add the following note to the table:

    Note: Each animal will receive a target dose of approximately 50 to 75 µCi/kg.

AMENDMENT APPROVAL

Gunda Reddy, PhD, DABT
Study Monitor
U.S. Army

10 August 2004
Date

Milan A. Berge, PhD
Study Director
Drug Metabolism
Covance Laboratories Inc.

06 August 2004
Date

Jon F. Denissen, PhD
Executive Director
Drug Metabolism
Covance Laboratories Inc.

09 August 2004
Date

COVANCE
THE DEVELOPMENT SERVICES COMPANY

PROTOCOL AMENDMENT NO. 2

Covance 7273-121

Absorption, Distribution, Metabolism, and Excretion of ^{14}C-RDX Following Oral Administration to Minipigs

Sponsor:	U.S. Army Center for Health Promotion and Preventive Medicine, Aberdeen Proving Ground, MD
Study Monitor:	Gunda Reddy, PhD, DABT
Study Location:	Covance Laboratories Inc., Madison, WI
Study Director:	Milan A. Berge, PhD

This amendment modifies the following portions of the protocol:

Effective 12 February 2010

1. **Page 1, Cover Page.** To reflect a change in study director, update the study director and location with the following (changes in bold):

Study Director and Study Location	**Timothy J. Musick, PhD** Covance Laboratories Inc. 3301 Kinsman Boulevard Madison, WI 53704-2523 Telephone: **608.241.7216** Facsimile: 608.241.7412 e-mail: **timothy.musick@covance.com**

AMENDMENT APPROVAL

Gunda Reddy, PhD, DABT
Study Monitor
U.S. Army

4-6-2010
Date

Timothy J. Musick, PhD
Study Director
Drug Metabolism
Covance Laboratories Inc.

13 April 2010
Date

Frederic W. Thalacker, PhD
Director
Drug Metabolism
Covance Laboratories Inc.

13 April 2010
Date

Protocol Deviations

Protocol Procedure	Actual Procedure
Approximately 500 g of Certified Diet #7037 (Harlen Teklad) will be provided twice daily.	Due to a delay in the study, starting on 21 July 2004, animals were provided approximately 300 g of food twice a day.
Approximately 500 g of Certified Diet #7037 (Harlen Teklad) will be provided twice daily.	Animals were fed PMI #5082 mini pig diet.
The protocol does not indicate that should emesis occur, vomitus will be collected.	Vomitus was collected from all animals.
Storage of the reference substances was provided in the protocol.	The storage for the TNX, MNX, DNX, and MEDINA prior to 29 January 2003 was not documented; therefore it cannot be verified.
The TNX and MEDINA will be stored at ambient temperature.	The TNX and MEDINA were stored in a freezer set to maintain –10 to –30°C.
The report will include amounts of radioactivity in tissues (tissues collected in their entirety), expressed as percent of the total administered dose.	The percent of radioactive dose was also reported for blood, muscle, and fat using Sponsor-supplied extrapolation values.
At 24 hours postdose (± 20 minutes), animals will be sacrificed via exsanguination under sodium pentobarbital anesthesia.	Animal M01644 was sacrificed 1 minute outside the acceptable time range.

In the opinion of the study director, these deviations would not be expected to affect the outcome of the study.

Appendix 2
Dose Formulation Analysis

HPLC System and Conditions

HPLC System

Pump:	HP 1100 Series
Autoinjector:	HP 1100 Series
Column heater:	HP 1100 Series
Degasser:	HP 1100 Series
UV detector:	HP 1100 Series
Wavelength:	236 nm
Radioactivity detector:	Packard 500 Series
Flow cell size:	0.5 mL TRLSC
Scintillation cocktail	Ultima Flo M
Scintillation cocktail flow rate:	3 mL/minute
Column:	Zorbax SB-C18, 4.6 x 250 mm, 5 μm
Column temperature:	25°C
Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	Acetonitrile
Flow rate:	1 mL/minute

Gradient:

Time (minutes)	A(%)	B(%)
Initial	98	2
5	98	2
30	30	70
31	98	2
40	98	2

Table 2-1
Dose concentration and homogeneity results

Group	Aliquot	Mean Concentration of Radioactivity (dpm/g by Aliquot)	Mean Concentration of Radioactivity (dpm/g formulation)	Specific Activity (μCi/mg)	Test Article Concentration (mg/g)
1	Predose	2.66×10^7	2.61×10^7	1.30	8.97
		2.57×10^7			
	Postdose	2.48×10^7	2.56×10^7		
		2.64×10^7			

Table 2-2
Dose stability determination

Group	Analysis	Radiopurity (%)	Column Recovery (%)
1			
	Predose	95.4	105
		95.3	105
	Mean	95.3	105
	Postdose	96.2	103
		96.0	103
	Mean	96.1	103

Figure 2-1
Representative chromatogram for the radiopurity of ^{14}C-RDX

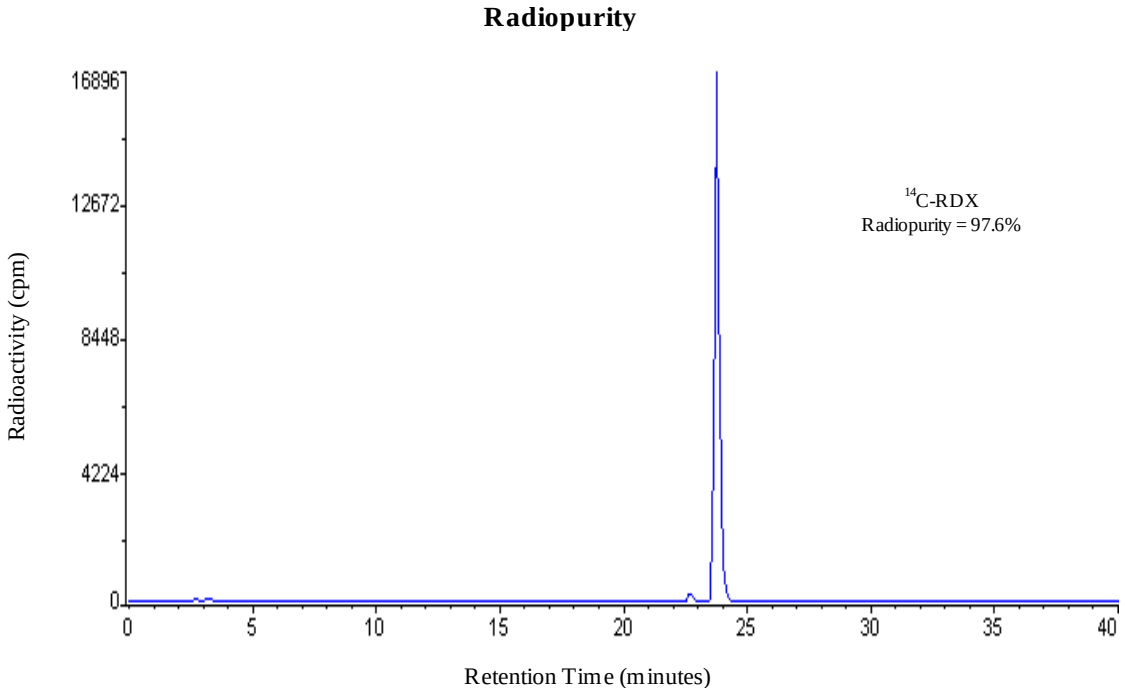
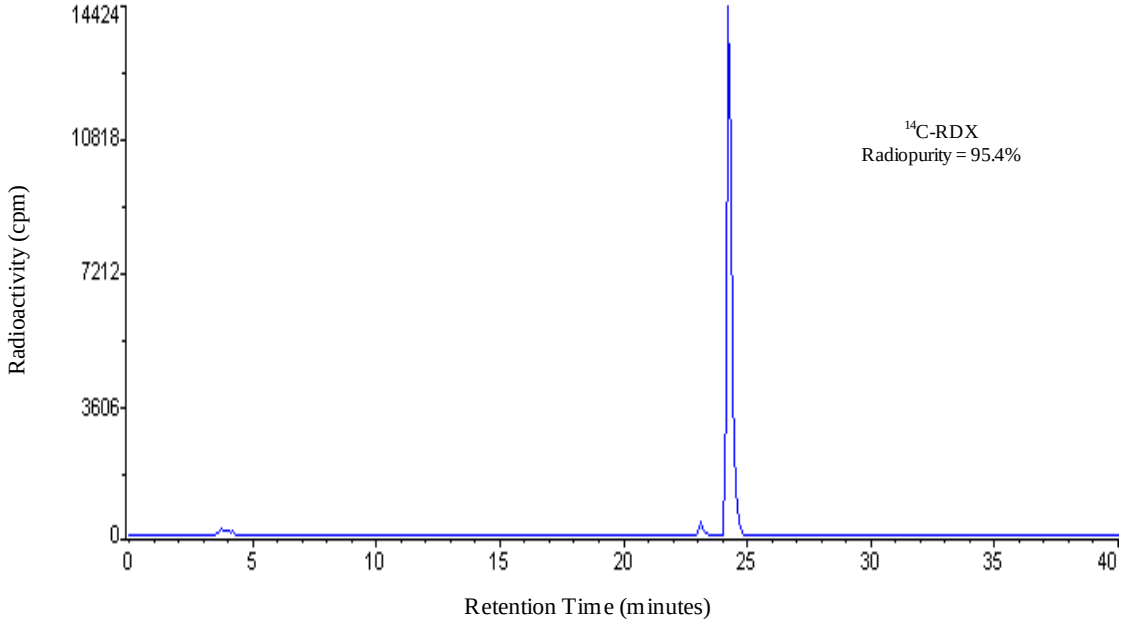
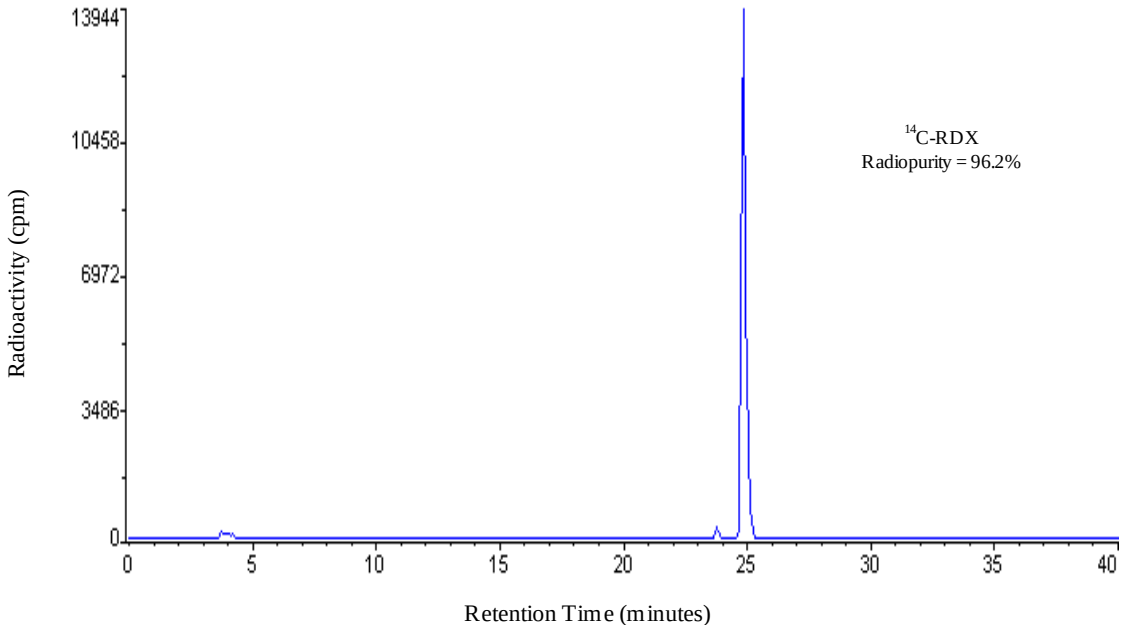


Figure 2-2
Representative chromatograms for predose and postdose analysis of the oral dose formulation

Predose



Postdose



Appendix 3
Calculation Formulas

Calculation Methods Used by Debra

Dose administered may be reported as amount of radioactivity or test article per unit of body weight. Calculations are done in Debra, with the exception of parameters that Debra does not calculate: μCi/kg of body weight, and values for mean, standard deviation, standard error of the mean, and coefficient of variation for dose administered. The parameters not calculated in Debra are calculated in Microsoft Excel.

The specific activity, concentration of test article in the dose formulation, and concentration of radioactivity in the dose formulation are used to calculate the dose administered. The amount of dose formulation administered is determined by weighing the full dosing syringe prior to dose administration and the emptied syringe after dosing. If a dose wipe or dose apparatus (labeled "site wipe" by Debra) is collected, the radioactivity in the site wipe is analyzed and subtracted from the radioactivity in the administered dose formulation. Example formulas follow.

$$Dose\ \ administered\ (g) = [Full\ syringe\ weight\ (g) - Empty\ \ syringe\ weight\ (g)] - (\frac{Site\ wipe\ dpm}{Dose\ dpm/g})$$

Radioactivity administered:

$$dpm/animal = Dose\ \ administered\ (g) \times Dose\ dpm/g$$

$$\mu Ci/animal = \frac{dpm/Animal}{2.22\ x\ 10^{6}\ dpm/\mu Ci}$$

$$\mu Ci/kg = \frac{\mu Ci/Animal}{Animal\ body\ weight\ (kg)}$$

Test article administered:

$$Test\ article\ administered\ (mg) = Dose\ administered\ (g) \times Dose\ concentration\ (mg/g)$$

$$mg/kg = \frac{Test\ article\ administered\ (mg)}{Animal\ body\ weight\ (kg)}$$

Concentrations of radioactivity in individual samples may be based on weight or volume. Weights are used in the following examples.

The dpm/unit weight calculation is common to all sample processing types. The following calculations are used.

$$dpm/g = \frac{Aliquot\ dpm - Background\ dpm}{Aliquot\ weight\ (g)}$$

$$Mean\ dpm/g = \frac{\sum (Aliquot\ dpm/g)}{n}$$

The inter-replicate variability, labeled in Debra as "variance," is the calculated coefficient of variation (%).

$$Variance = \frac{Standard\ deviation\ (dpm/g)}{Mean\ dpm/g} \times 100$$

If this value is greater than 10%, the sample is flagged as high and analysis is repeated if there is a sufficient amount of sample remaining.

For some samples, water or a solvent may be added to dilute the sample, extract the radioactivity, or facilitate homogenization. The following calculations are used, where homogenate weight is the combined weight of the sample and the solvent.

$$\mu g\ Equivalents/g = \frac{Mean\ dpm/g \times Sample\ \ weight\ or\ Homogenate\ weight\ (g) \times 1000\ \mu g/mg}{Specific\ activity\ (\ \mu Ci/mg) \times 2.22\ x\ 10^{6}\ (dpm/\mu Ci) \times Sample\ weight\ (g)}$$

$$dpm\ \ in\ Sample = Mean\ dpm/g \times Sample\ weight\ (g)\ or\ Homogenate\ weight$$

The calculation for dpm in Sample occurs within the system; the result is not reportable.

$$Percent\ of\ \ dose\ in\ sample = \frac{dpm\ in\ Sample}{dpm\ \ Administered} \times 100$$

For samples where there are only aliquot weights, the following calculations are used.

$$\mu g\ Equivalents/g = \frac{Mean\ dpm/g \times 1000\ \mu g/mg}{Specific\ activity\ (\ \mu Ci/mg) \times 2.22 \times 10^{6}\ (dpm/\mu Ci)}$$

$$Percent\ dose = \frac{\sum Aliquot\ dpm}{dpm\ \ Administered} \times 100$$

Where: $\sum$Aliquot dpm = the sum of dpm detected in all sample aliquots.

Blood, muscle, and fat, which were not collected in their entirety, were scaled to total body composition. These extrapolation values were taken from the following PhD thesis in consultation with the Sponsor.

L.B. Sasser, "The relationship of Whole Body Counting of Potassium 40 to Body Composition of Swine," Colorado State University, (1968).

Values for these tissues are as follows:

Tissue	% of Pig Body Weight
Blood	6
Muscle	36.7
Fat	22.1

Debra Summary Tables

All calculations are performed on unrounded numbers. Values reported by Debra are calculated directly from raw data values each time a report is generated. Slight variation may occur between intermediate and summary table values if different parameters were specified when these tables were generated. Final report tables reflect the summary tables.

Concentration values are reported to three significant figures. Percent of dose is reported to three significant figures with a maximum of two decimal places. Calculation of the mean includes all decimal places. Therefore, in some instances, the reported mean value may differ slightly from the mean as calculated from individual reported values. Expressions of variance are not reported to a greater degree of accuracy than the mean. Standard deviation and standard error of the mean are generally reported to the same number of decimal places as the mean, with a maximum of three significant figures. For very large numbers, standard deviation and standard error of the mean are reported to the same place (ones, tens, hundreds, etc.) as the mean. Coefficient of variation, or relative standard deviation, is reported to the nearest whole number. If the calculated percent of dose is <0.005, the value is reported as 0.00.

If results of sample analysis are below twice background, Debra reports the result as less than the limit of quantitation (BLQ). Values of BLQ are included as 0 in calculations of mean and variance. The concentration associated with the limit of quantitation is calculated as follows.

$$Limit\ of\ Quantitation(\ \mu g\ equivalents\ /\ g\ ) = \frac{2 \times Background\ (\ dpm\ ) \div Aliquot\ weight\ (\ g\ )}{Dose\ specific\ activity\ (\ dpm\ /\ \mu g\ )}$$

The percent of administered dose associated with the limit of quantitation is calculated as follows.

$$Limit\ of\ Quantitation\ (\%\ of\ \ dose) = \frac{Limit\ of\ \ quantitation\ (\ \mu g\ equivalents/g) \times Sample\ weight\ (g)}{\mu g\ administered} \times 100$$

Appendix 4
LC/MS Conditions and Spectra

LC/MS Analysis of Isolated Urine Peaks (Method 1)

LC/MS Instrumentation and Software

LC system controller:	Shimadzu, Model SCL-10A VP
Pumps A and B:	Shimadzu, Model LC-10AD VP
Injector:	Shimadzu, Model SIL-10AD VP
HPLC column:	Zorbax SB-CN 5 µM, 150 x 4.6 mm i.d.
Degasser:	Shimazdu DGU-14A
Column oven:	Shimadzu, Model CTO-10AC VP (35°C)
Valve switch:	Shimadzu, Model CTO-10AC VP
Mass spectrometer:	Applied Biosystems Qtrap-4000 with Turbo Spray source
Radiochemical detector:	Radiomatic Series 500 (Packard)
Mass spectrometer software:	Analyst 1.4 (Applied Biosystems)
Radiochemical detector software:	Flo-One Version 3.65 (Packard)

Solvent Program

Time (minutes)[a]	% A	% B	Curve
Initial	97	3	NA
1.5	97	3	Hold
2.0	50	50	Linear
4.0	50	50	Hold
4.1	97	3	Linear
5.0	97	3	Re-equilibration

NA Not applicable.
a Elapsed time.

Solvent A: 0.02% acetic acid in water
Solvent B: 0.02% acetic acid in methanol
HPLC flow was split to allow about 50% to mass spectrometer and 50% to the radiomatic detector.

HPLC flow rate: 1.0 mL/minute
LSC flow rate: 1.5 mL/minute (Ultima-FloM LSC cocktail, Packard)

To minimize contamination of the mass spectrometer source, the HPLC effluent of the first 2.20 minutes of each run was diverted to waste using the switching valve.

Negative Ion LC/MS/MS Analyses

Selective multiple reaction monitoring (MRM) was used as survey scan to activate product ion scans (MS2) through preset information dependent acquisition (IDA) criteria. Q1 full scan, precursor scan (prec) of specific product ions at m/z 61 and 46, as well as product ion scan (MS2) were used to detect and confirm the existence of metabolites M1 and M2. The following standards or samples were analyzed using these methods.

Sample	Injection Volume (µL)	MS Data File
Mixed Standards[a]	50	DataSet 020805.wiff
Urine Peak #1	50	DataSet 020805.wiff
Urine Peak #2	50	DataSet 021005C.wiff
		DataSet 021005E.wiff
		DataSet 021005F.wiff

a Mixture of reference standards of 4-nitro-2,4-diazabutanal and methylenedinitramine at 1.00 µg/mL.

The mass spectrometer conditions were:

Ionization mode:	Turbo Spray negative
Ion Spray voltage:	-1500 v
Mass range:	Variable
Scan time:	0 to 5 minutes
Collision Energy (CE):	Variable
Source temperature:	400°C
CAD Gas:	High (nitrogen)
Curtain Gas:	40 psi (nitrogen)
Nebulizer Gas (GS1):	30 psi (nitrogen)
Turbo Gas (GS2):	30 psi (nitrogen)
Declustering Potential (DP):	-22 v

LC/MS Analysis of Plasma and Urine Samples (Method 2)
Full-Scan Analyses
LC/MS Instrumentation and Software

LC system controller:	Shimadzu, Model SCL-10A VP
Pumps A and B:	Shimadzu, Model LC-10AD VP
Injector:	Shimadzu, Model SIL-10AD VP
HPLC column:	Agilent ZORBAX SB-C18, 5µm, 250 mm x 4.6 mm i.d.
Guard column:	Phenomenex C18, 3 mm x 4.0 mm i.d.
Column oven:	Shimadzu, Model CTO-10AS VP (25°C)
UV detector:	Shimadzu, Model SPD-10A (236 nm)
Valve switch:	Shimadzu, Model FCV-12AH
Radiochemical detector:	Radiomatic Series 500 (Packard)
Radiochemical detector software:	Flo-One Version 3.65 (Packard)
Mass spectrometer:	Micromass Quattro II with an ESP Z-Spray source
Mass spectrometer software:	MassLynx Version 3.4 (Micromass)

Solvent Program

Time (minutes)[a]	% A	% B	Curve
Initial	98	2	NA
5	98	2	Hold
30	30	70	Linear
31	98	2	Linear
40	98	2	Re-equilibration

NA Not applicable.
a Elapsed time.

Solvent A: 0.05% formic acid in reverse-osmosis water
Solvent B: Acetonitrile

HPLC flow rate: 1.0 mL/minute
LSC flow rate: 2.1 mL/minute (Ultima-FloM LSC cocktail, Packard)

After passing through the column switcher, the HPLC column effluent was split with approximately 30% of the flow diverted to the mass spectrometer and 70% to the

radiometric detector. To minimize contamination of the mass spectrometer source, the first 3.5 minutes of each run was diverted to waste using the switching valve.

The mass spectrometer conditions were:

Ionization mode:	ESP negative
Cone voltage:	12 volts
Mass range:	40 to 500 amu
Scan time:	0 to 35 minutes
Source block temperature:	130°C
Desolvation temperature:	300°C
ESI nebulizer gas:	15 L/hr (nitrogen)
Bath gas:	400 L/hr (nitrogen)

The mass spectrometer was tuned daily using the mobile phase ions. This standard procedure ensured that optimum conditions for signal stability and sensitivity were met.

The following standards and sample were analyzed using negative ion full-scan LC/MS Method 2:

Sample	Injection Volume (µL)	MS Data File
Standard MS-1[a]	10	04040501
Male M01638 12 hour plasma	100	04040504

a Mixture of authentic standards of RDX, MNX, DNX and TNX.

Product Ion Analyses

Based on the full-scan LC/MS data, a number of product ion analyses were performed for structural elucidation. The product analyses used the same instrumentation and conditions as for full-scan LC/MS, with the following exceptions to the mass spectrometer conditions:

Collision energy:	5 or 7 eV
Mass range:	Variable
Scan time:	Variable
Collision gas:	Argon

Multiple Reaction Monitoring (MRM) Analyses

Analysis of selected plasma samples using MRM was performed to determine the metabolite composition of each sample. Analysis using the MRM technique was chosen because it allowed for greater selectivity than full-scan or single ion recording (SIR) techniques. The precursor and product ions shown in the table below were chosen for the analyses based on the product ion analyses of standards and study samples (above). The MRM method used the same instrumentation and conditions as for the full-scan LC/MS analyses, with the following exceptions to the mass spectrometer conditions:

- Dwell time: 0.2 seconds
- Collision gas: Argon

Component	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (eV)
RDX	267	46	7
RDX	267	92	5
MNX	251	46	5
DNX	235	46	7
TNX	219	45	7

The following standards and plasma and urine samples were analyzed using MRM:

Sample	Injection Volume (µL)	MS Data File
Standard MS-1[a]	10	04040503
M M01638 12 hour plasma	100	04040506
M M01638 1 hour plasma	100	04040508
M M01638 6 hour plasma	100	04040509
M M01640 1 hour plasma	100	04040510
M M01640 6 hour plasma	100	04040511
M M01640 12 hour plasma	100	04040512
Standard MS-1[a]	10	04040513
Standard MS-1[a]	10	08080501
Standard MS-1[a]	10	08080502
M M01638 pooled urine	25	08080503
F M01644 pooled urine	50	08080504

a Mixture of authentic standards of RDX, MNX, DNX and TNX.

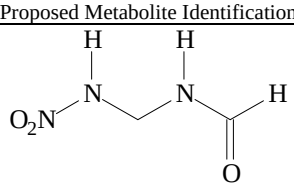
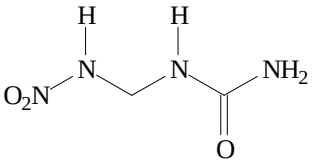
RESULTS

The chromatograms and mass spectra for urine analysis are presented in Figures 4-1 through 4-8. The product ion spectra of the 4-nitro-2,4-diazabutanal standard and methylenedinitramine are presented in Figures 4-9 and 4-10, respectively. The extracted ion chromatograms from analysis of a solution of standards utilized for plasma analyses are presented in Figure 4-11. The associated product ion mass spectra appear in Figures 4-12 through 4-15. The MRM chromatograms from analysis of a solution of authentic standards are presented in Figure 4-16. MRM chromatograms and radiochromatograms for plasma and urine analyses are presented in Figures 4-17 through 4-24. Mass spectra for plasma analyses are presented in Figures 4-25 through 4-27.

Isolated Urine Peaks (Method 1)

Metabolite M1 was first detected in Urine Peak 1 with MRM survey scans of *m/z* 118> 61 and 118>46. The IDA activated product ion scan triggered by these MRM survey confirmed the identity of M1 based on the direct comparison of retention times and product ion spectra between M1 and the standard (Figures 4-1 and 4-2). The radiomatic

detector also showed the peak eluting at 2.30 minutes, agreeing well with the MS detector. Metabolite M2 was initially detected in Urine Peak 2 with precursor (of *m/z* 61 and 46) scans (Figures 4-3 and 4-4). Its pseudo-molecular ion was determined to be *m/z* 133 (Figure 4-5). IDA activated product ion scans triggered by MRM survey scans of *m/z* 133>61 and 133>46 were again conducted in an effort to acquire product ion spectrum. The MRM survey scans confirmed the existence of M2 (Figure 4-7), but the product ion spectrum was severely interfered with a high background of *m/z* 97 and 133 (proposed as phosphoric acid and its di-water adduct, respectively) (Figure 4-8). Since this compound generates product ions at *m/z* 61 and 46, both specific for NO_2 group-containing standards of 4-nitro-2,4-diazabutanal (Figure 4-9) and methylenedinitramine (Figure 4-10); therefore, it must be related with the parent compound RDX and hence it is a metabolite. A summary of HPLC retention times, deprotonated molecular ions, sample origin, characteristic product ions, and identified metabolites follows.

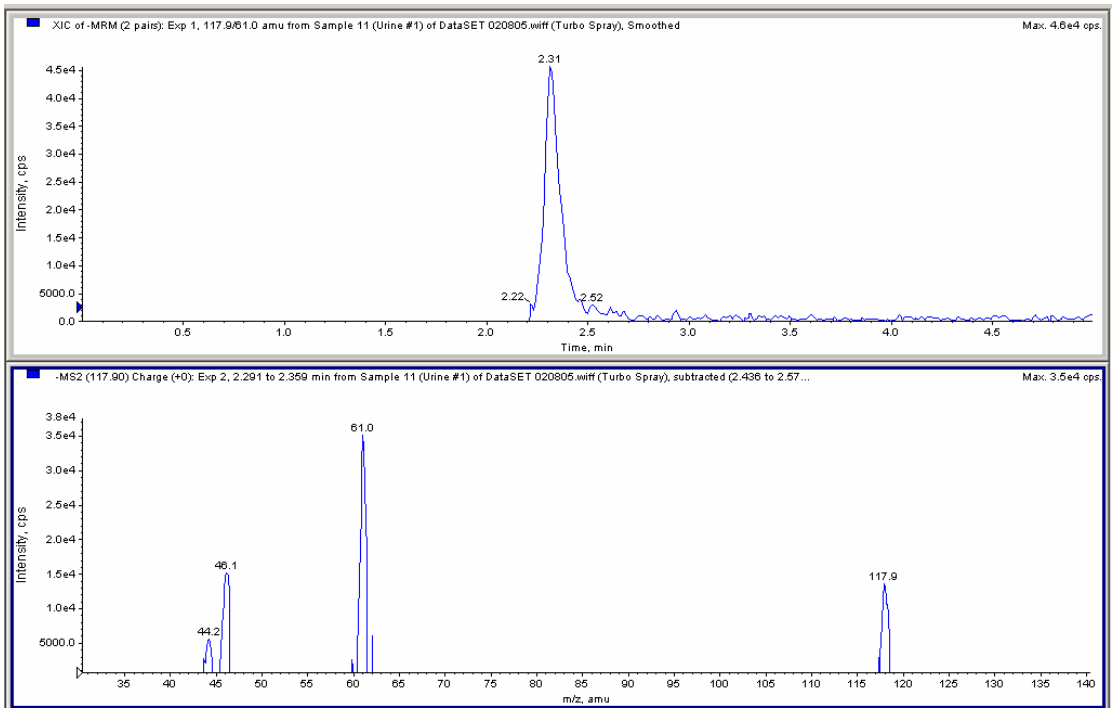
Metabolite	Retention Time (Minutes)	$[M - H]^-$	Proposed Metabolite Identification	Characteristic Product Ions (m/z)	Sample Origin
M1	2.35	118		**61**, 46, 44.	Urine peak 1
M2	2.26	133		**61**, 59, 46.	Urine peak 2

Plasma and Urine Samples (Method 2)

Plasma samples were analyzed using LCMS Method 2 in order to determine if RDX and its primary metabolites/degradates MNX, DNX, and TNX were present in study samples. Representative extracted ion chromatograms of the formate adduct $[M + HCO_2]^-$ of RDX, MNX, DNX, and TNX from analysis of a solution of authentic standards is in Figure 4-11. Representative product ion mass spectra of the formate adducts $[M + HCO_2]^-$ of the four authentic standards are shown in Figures 4-12 through 4-15. Based on the product ion spectra, an MRM method was developed to monitor the four components. The MRM chromatograms from analysis of a solution of authentic standards are presented in Figure 4-16.

The MRM chromatograms and radiochromatograms from analysis of the study plasma and urine samples are in Figures 4-17 through 4-24. These data verify the presence of RDX based on the fact that the MRM channels 267>192 and 267>46 cochromatograph with the major radioactive peak. Trace amounts of MNX, DNX, and TNX were detected in all plasma samples analyzed. Trace amounts of MNX, and DNX were detected in the male urine sample. Trace amounts of MNX were detected in the female urine sample. The presence of DNX, MNX, and RDX in study samples was confirmed by product ion analysis (Figures 4-25 through 4-27).

Figure 4-1
MRM survey chromatogram and product ion spectrum of metabolite M1 in urine Peak 1 (Method 1)



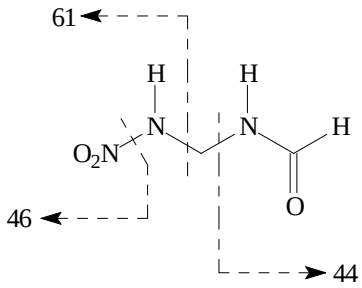
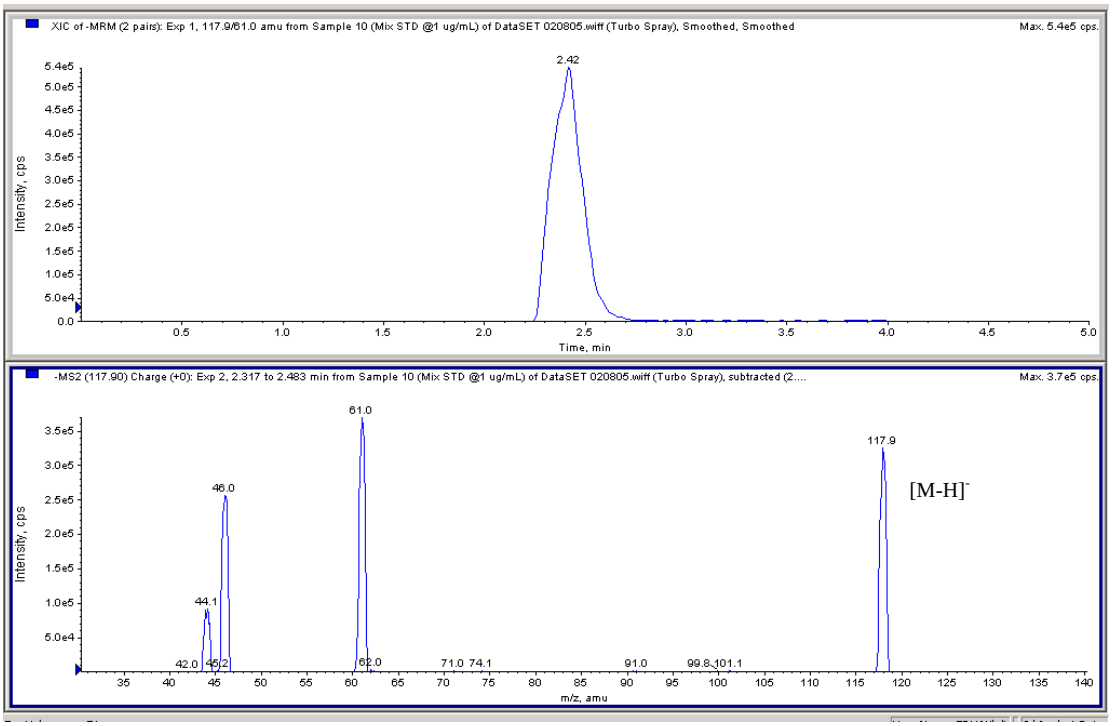


Figure 4-2
MRM survey chromatogram and product ion spectrum of 4-nitro-2,4-diazabutanal standard (Method 1)



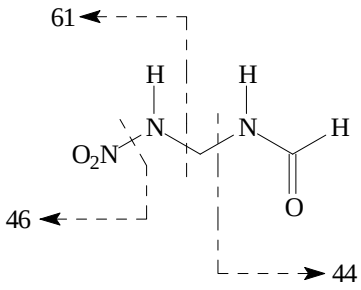


Figure 4-3
Precursor of m/z 61 chromatogram for detection of metabolite in urine Peak 2 (Method 1)

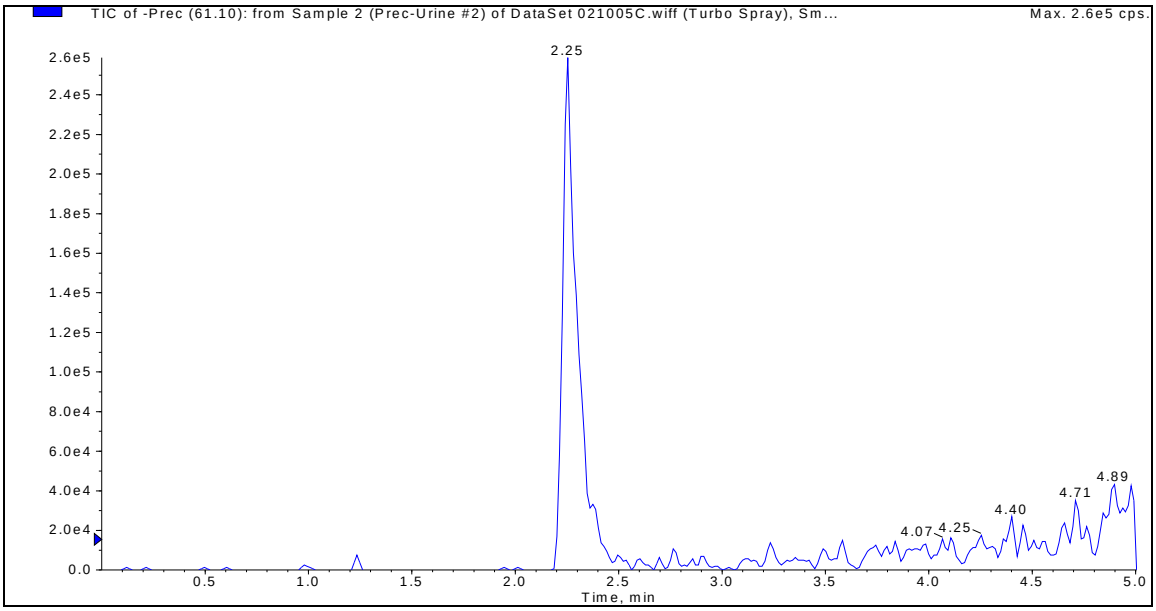


Figure 4-4
Precursor of m/z 46 chromatogram for detection of metabolite in urine Peak 2 (Method 1)

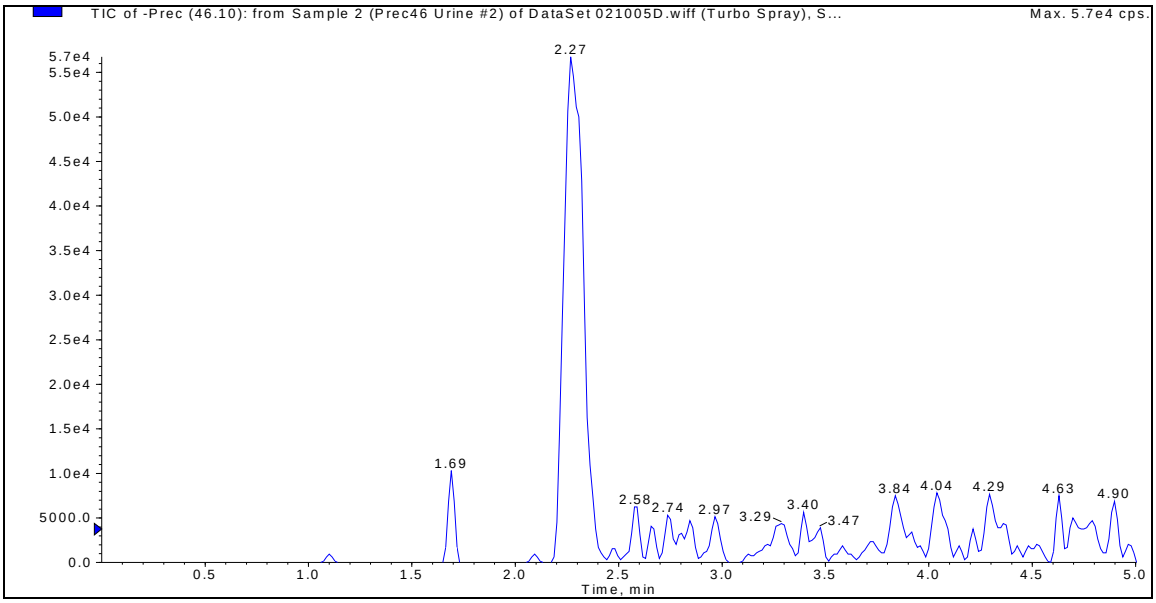


Figure 4-5
Chromatograms of pseudo-molecular ion determination of M2 in urine Peak 2 from both precursor of m/z 46 and of m/z 61 (Method 1)

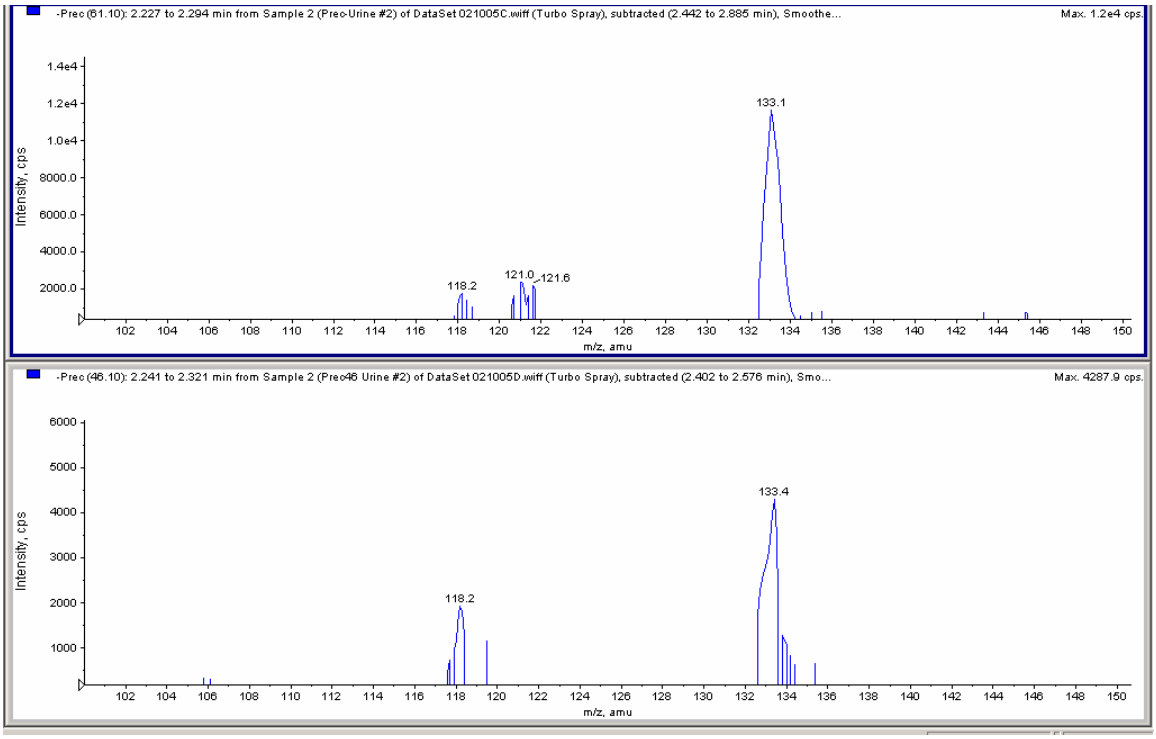
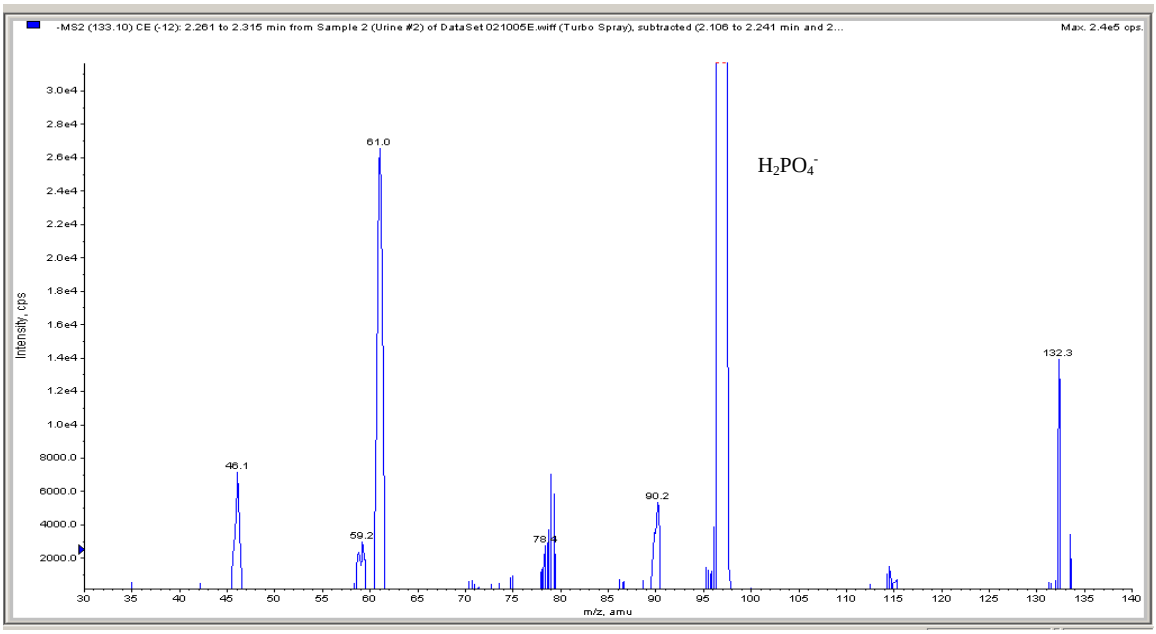


Figure 4-6
Product ion spectrum of m/z 133 at the retention time of 2.30 minutes of urine Peak 2 (Method 1)



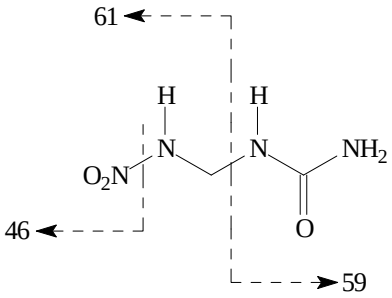
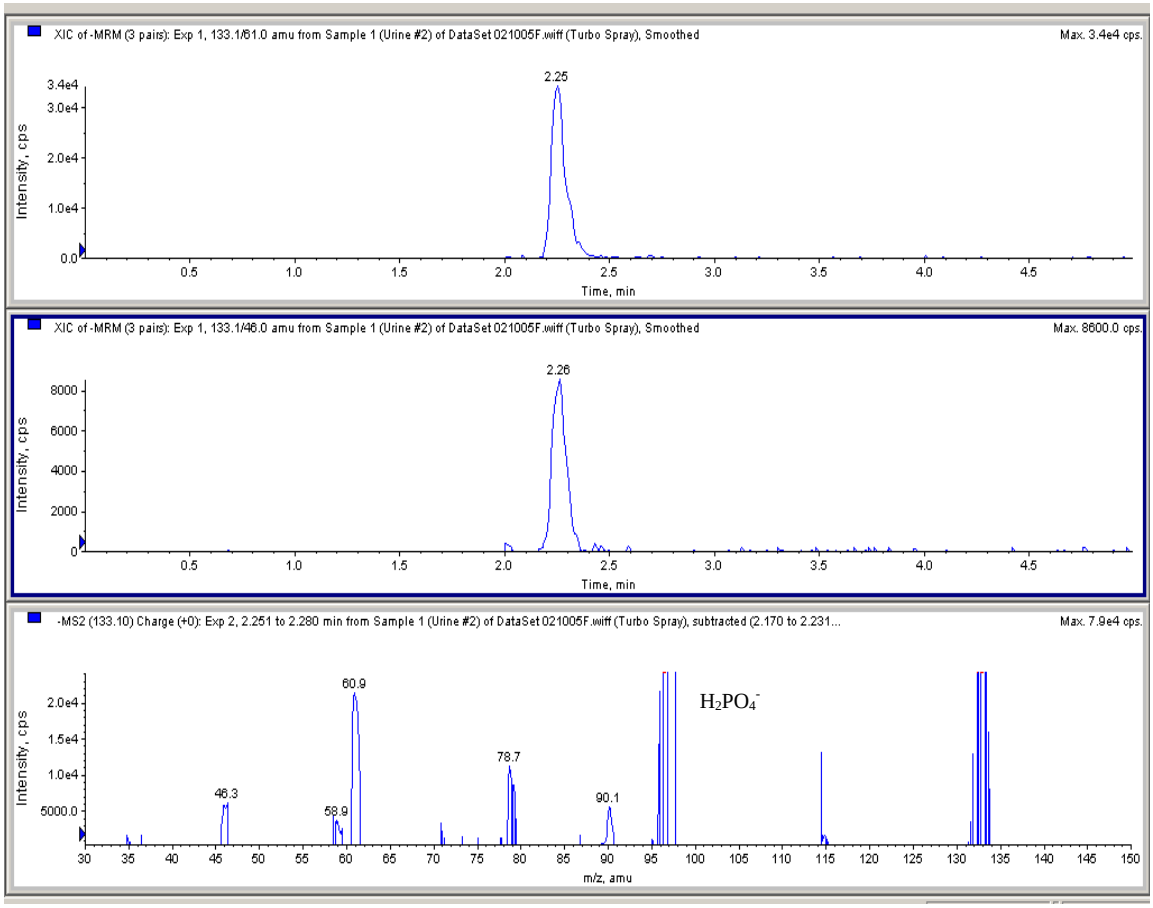


Figure 4-7
MRM survey chromatograms and product ion spectrum for confirmation of M2 in urine Peak 2 (Method 1)



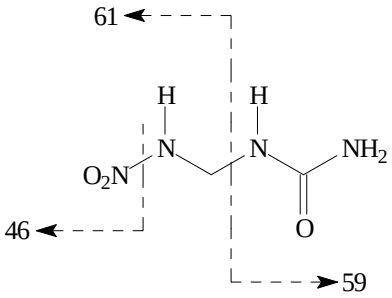


Figure 4-8
Product ion spectrum of M2 and background of urine Peak 2 (Method 1)

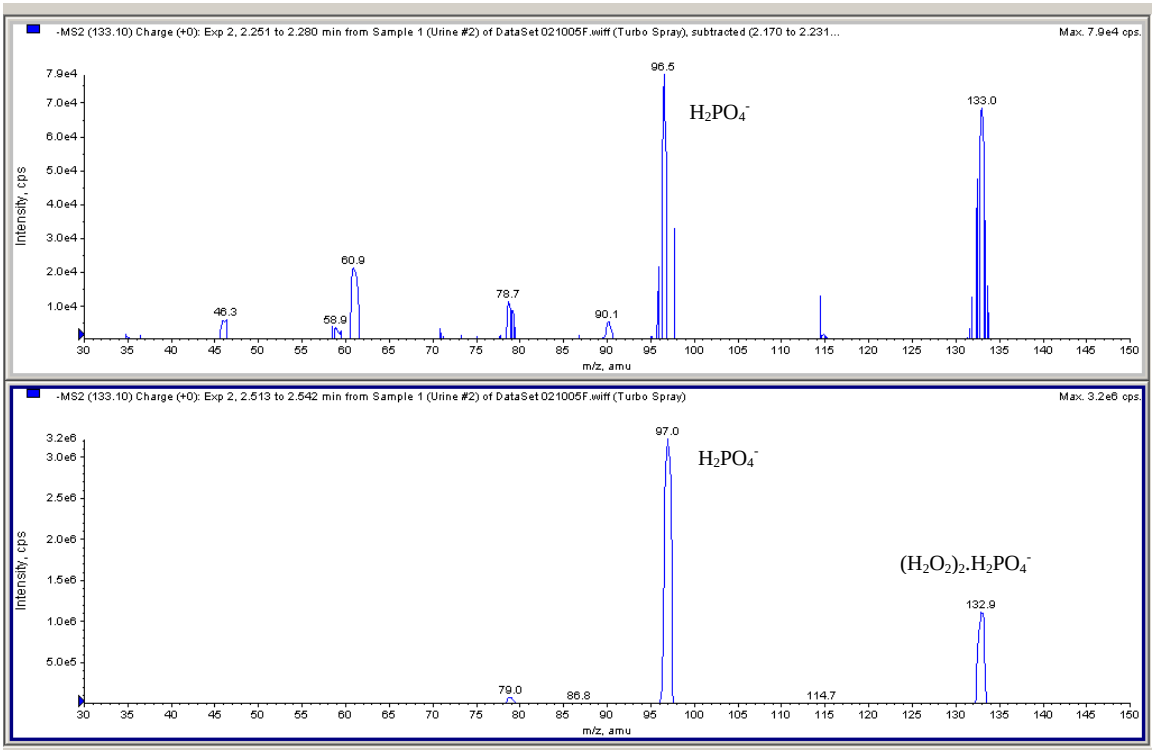
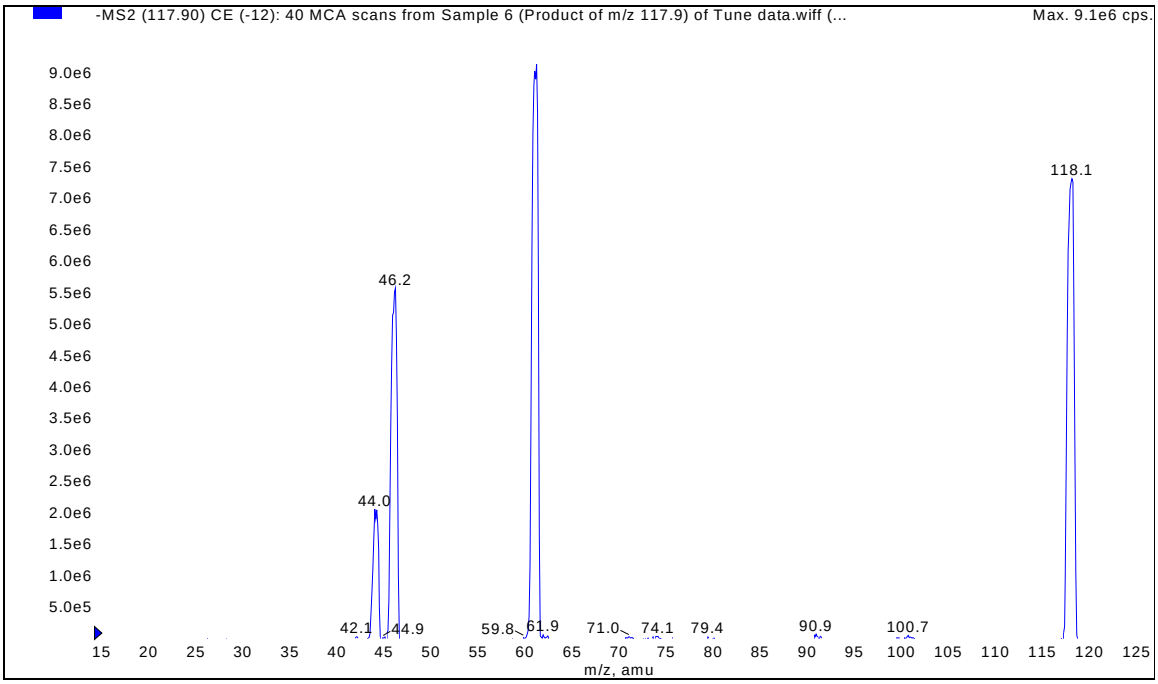


Figure 4-9
Product ion spectrum of 4-nitro-2,4-diazabutanal standard (Method 1)



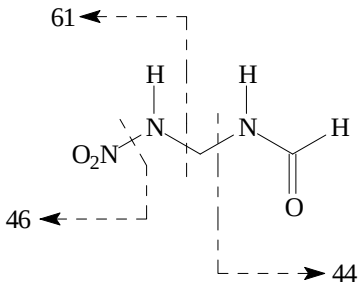
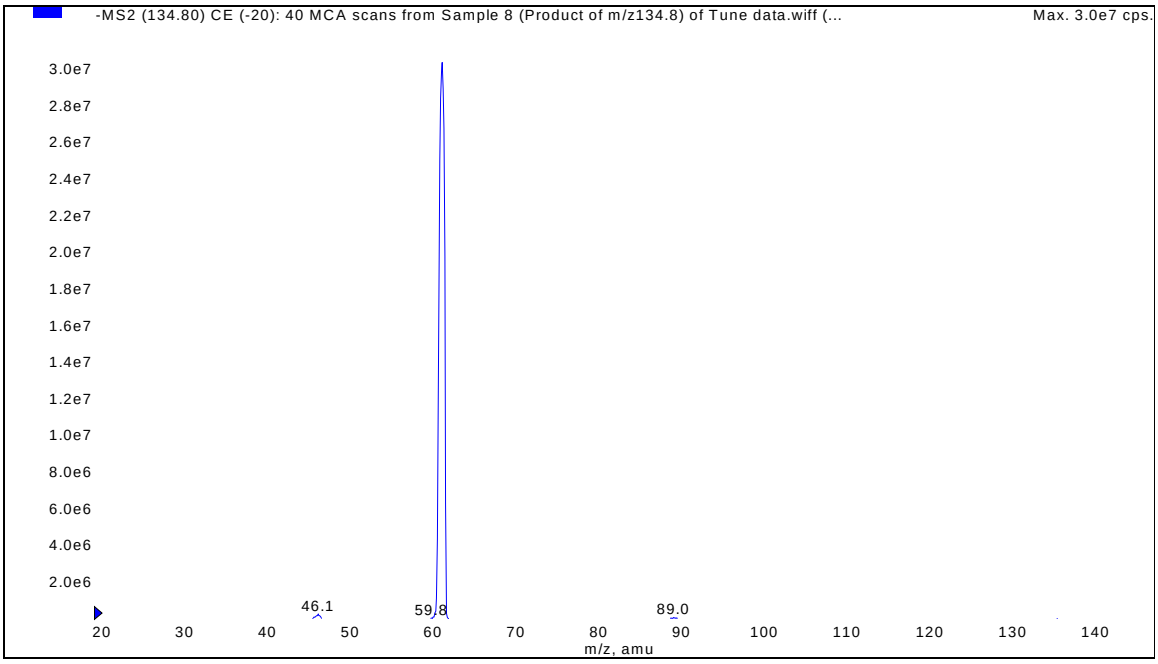


Figure 4-10
Product ion spectrum of methylenedinitramine (MEDINA) (Method 1)



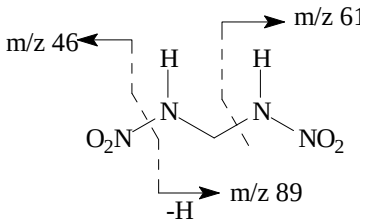
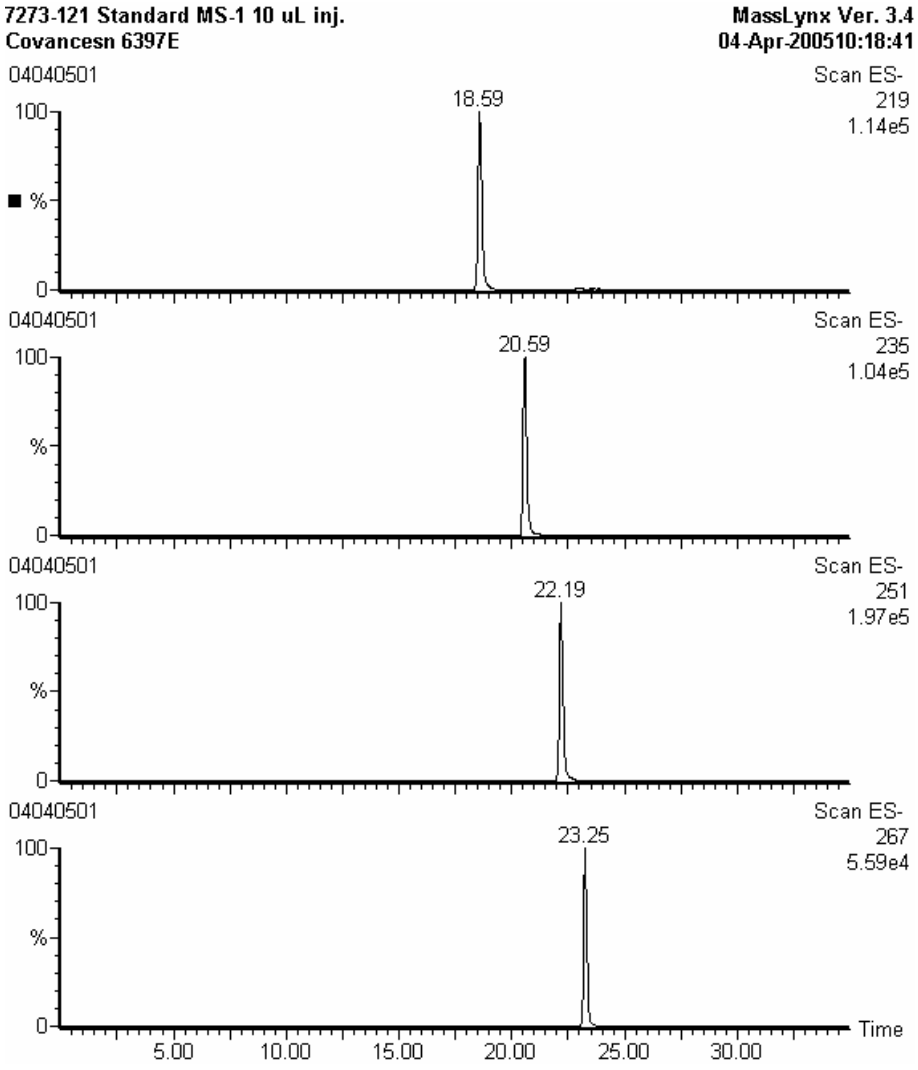


Figure 4-11
Extracted ion chromatograms of m/z 219, 235, 251, and 267 from analysis of a solution of standards using negative ion full-scan LC/MS Method 2



Component	Retention Time (minutes)	$[M + HCO_2]^-$
TNX	18.59	219
DNX	20.59	235
MNX	22.19	251
RDX	23.25	267

Figure 4-12
Product ion (m/z 219) mass spectrum of TNX from analysis of a solution of standards using LC/MS Method 2

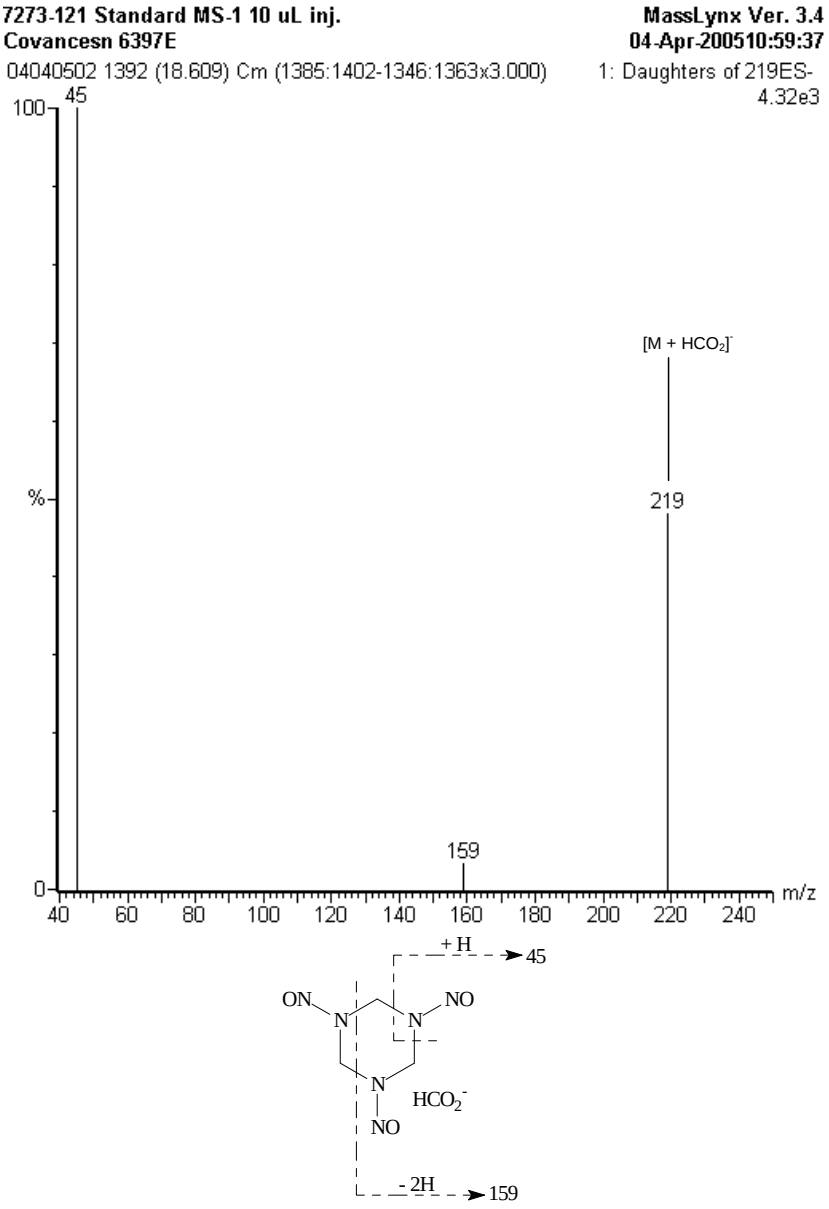


Figure 4-13
Product ion (m/z 235) mass spectrum of DNX from analysis of a solution of standards using LC/MS Method 2

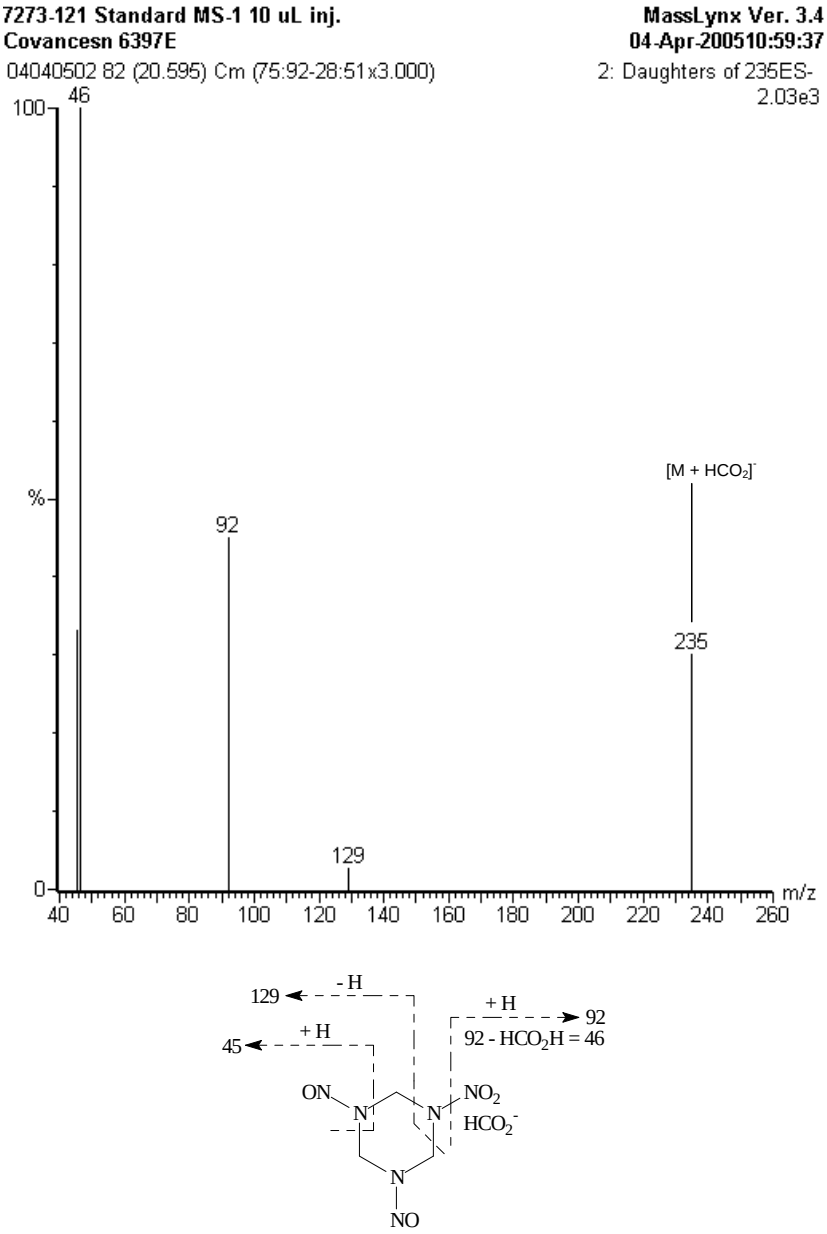


Figure 4-14
Product ion (m/z 251) mass spectrum of MNX from analysis of a solution of standards using LC/MS Method 2

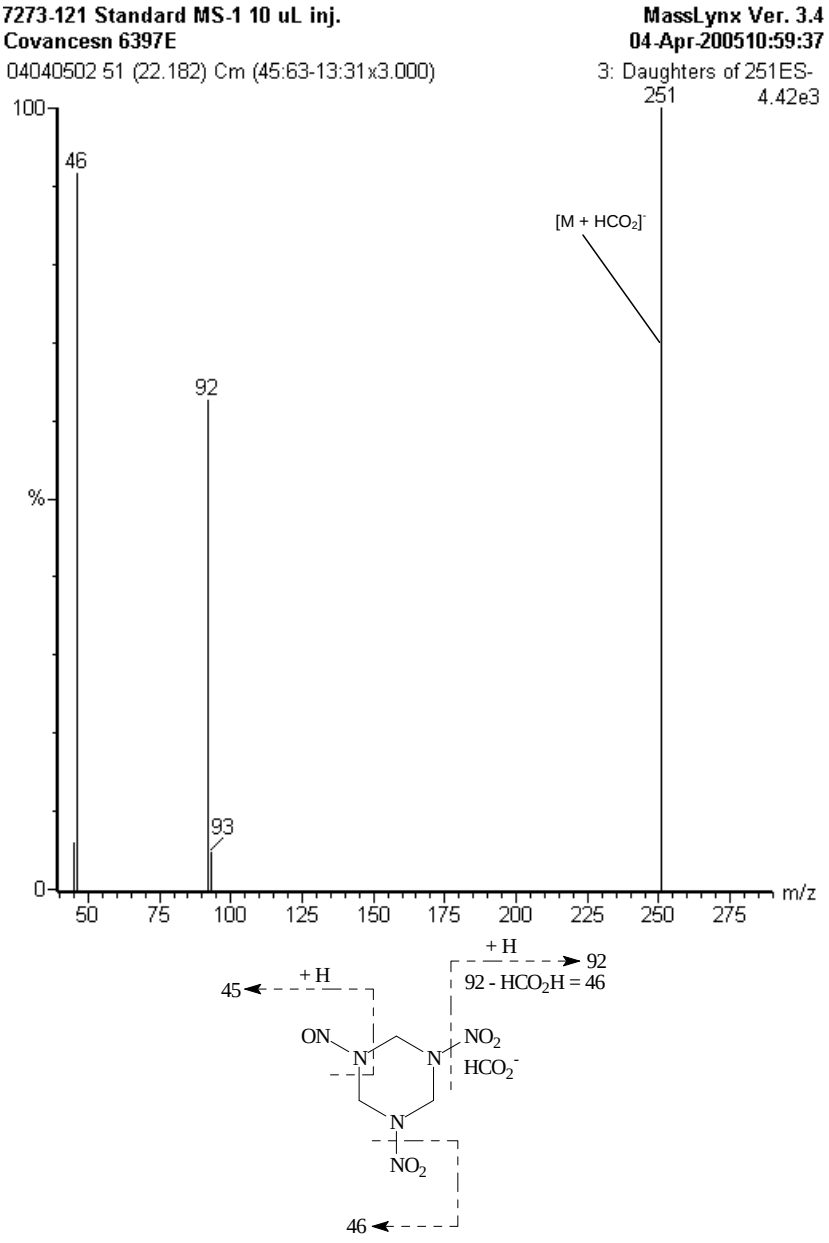


Figure 4-15
Product ion (m/z 267) mass spectrum of RDX from analysis of a solution of standards using LC/MS Method 2

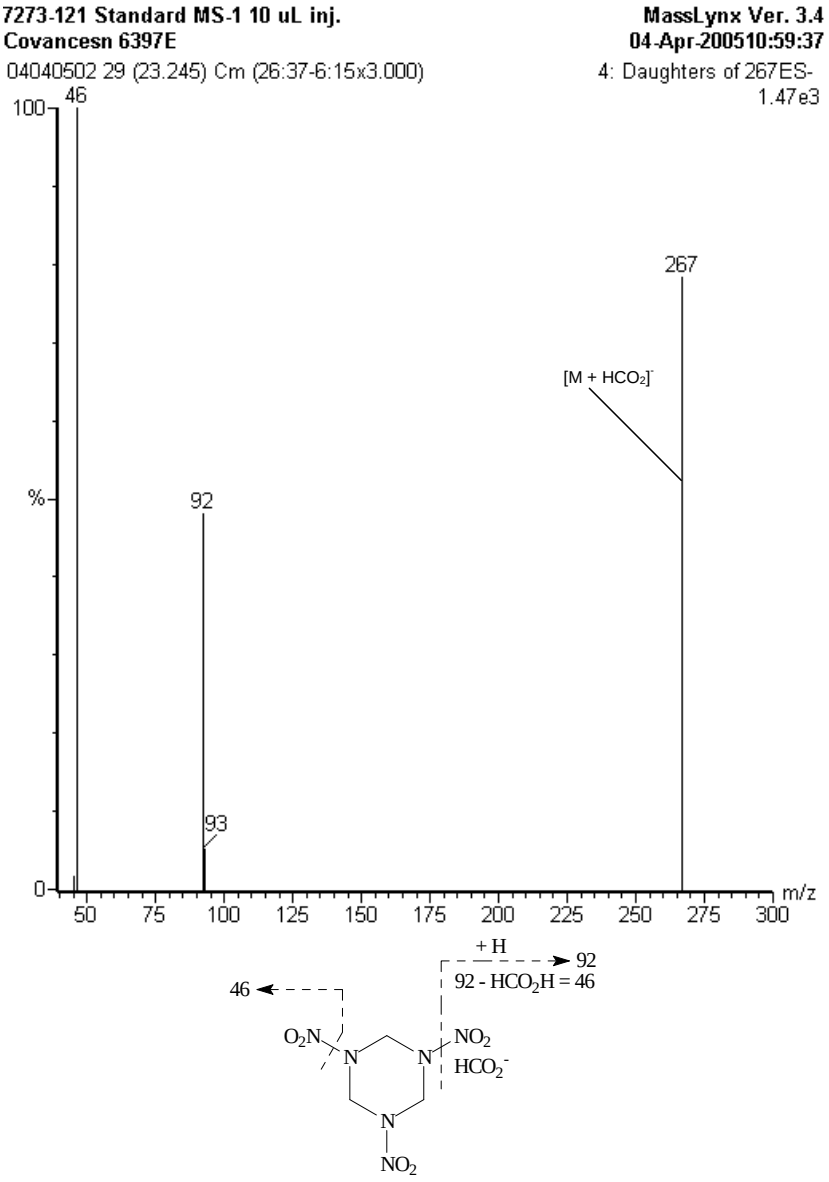
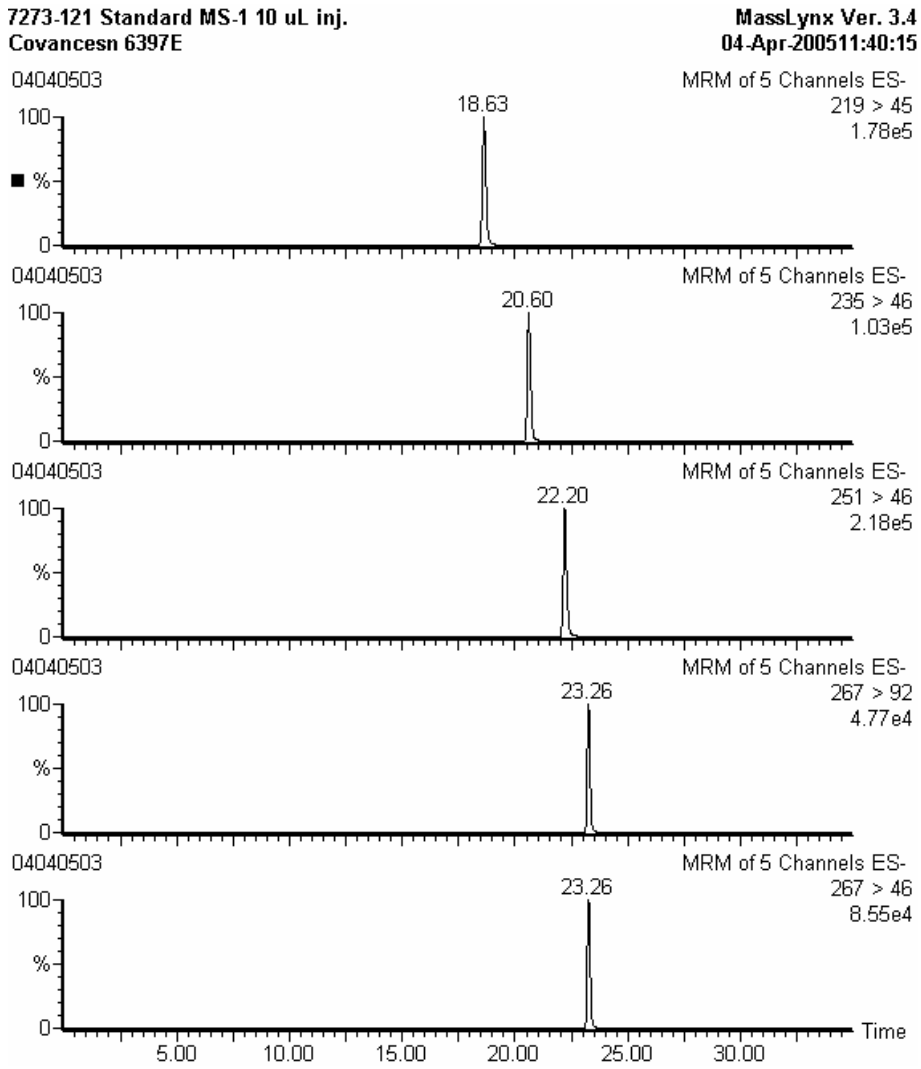
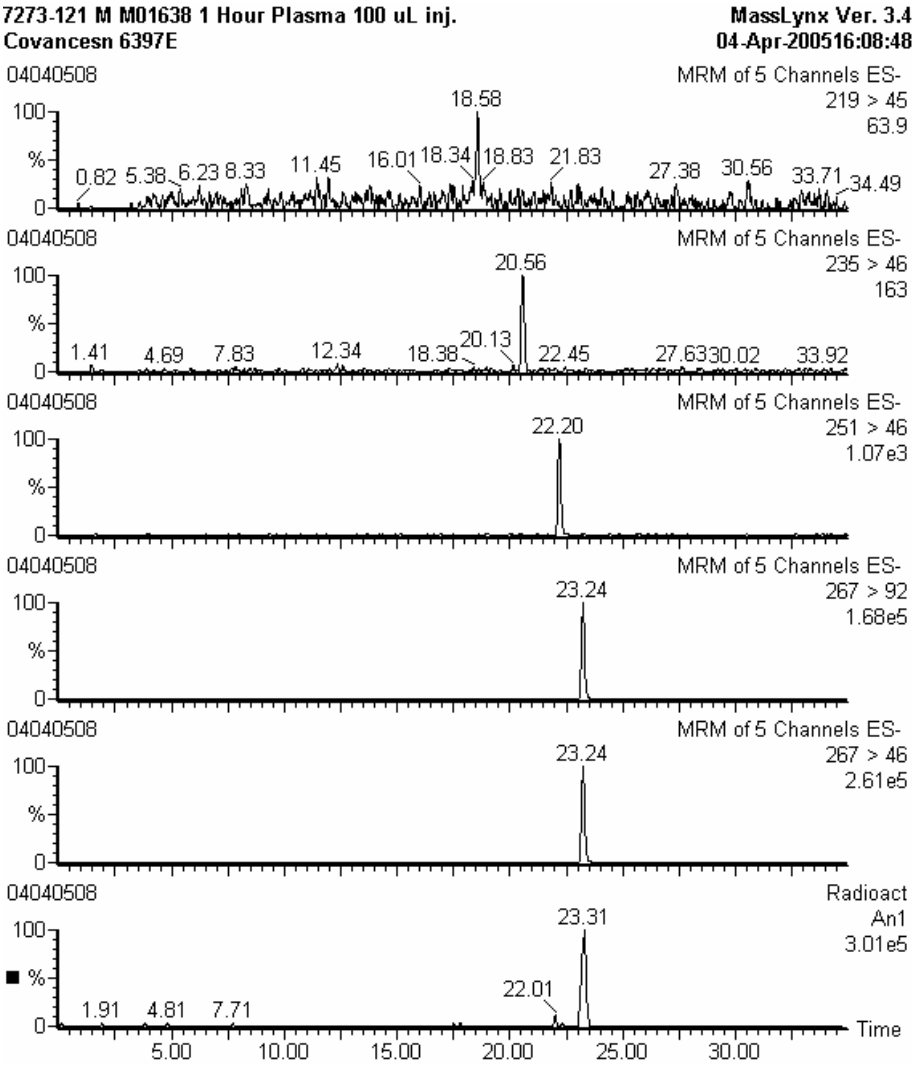


Figure 4-16
MRM chromatograms from analysis of a solution of standards using LC/MS Method 2



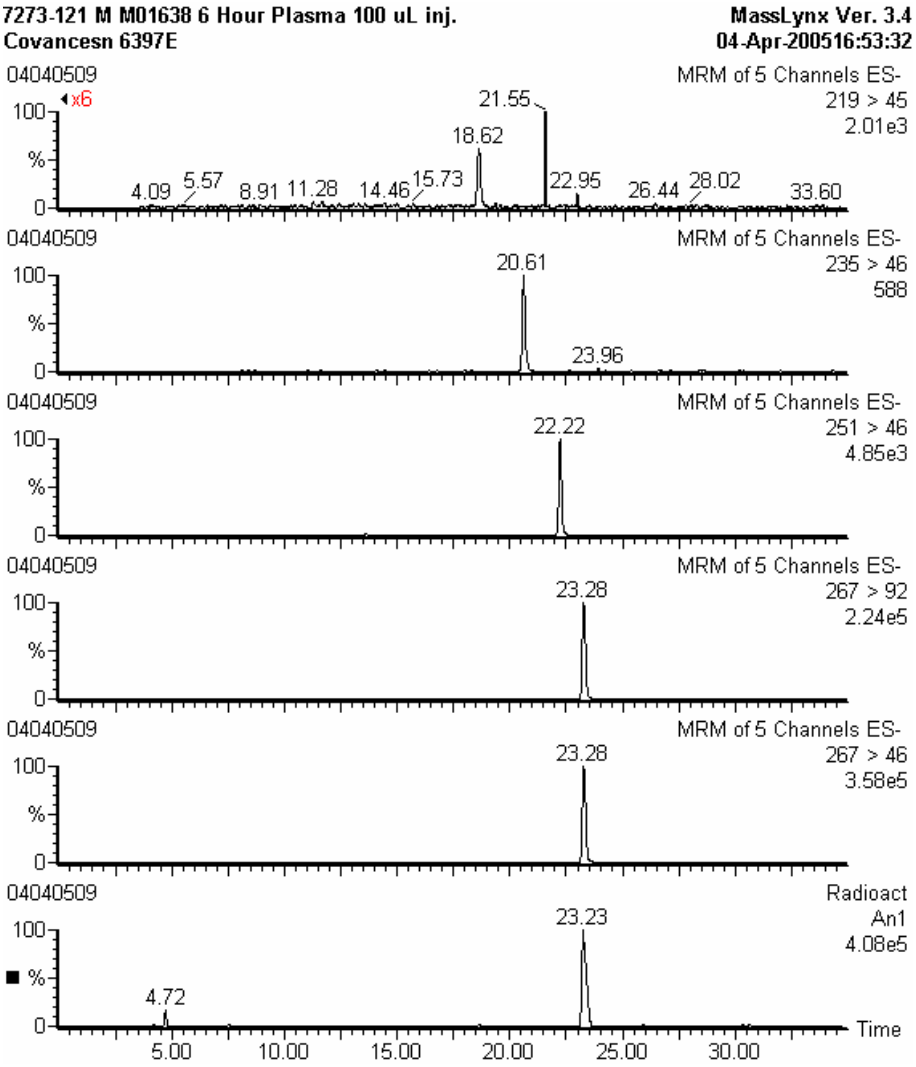
Component	Retention Time (minutes)	MRM Transition
TNX	18.63	219>45
DNX	20.60	235>46
MNX	22.20	251>46
RDX	23.26	267>92
RDX	23.26	267>46

Figure 4-17
MRM chromatograms and radiochromatogram from analysis of a 1 hour plasma sample from male Animal M01638 using LC/MS Method 2



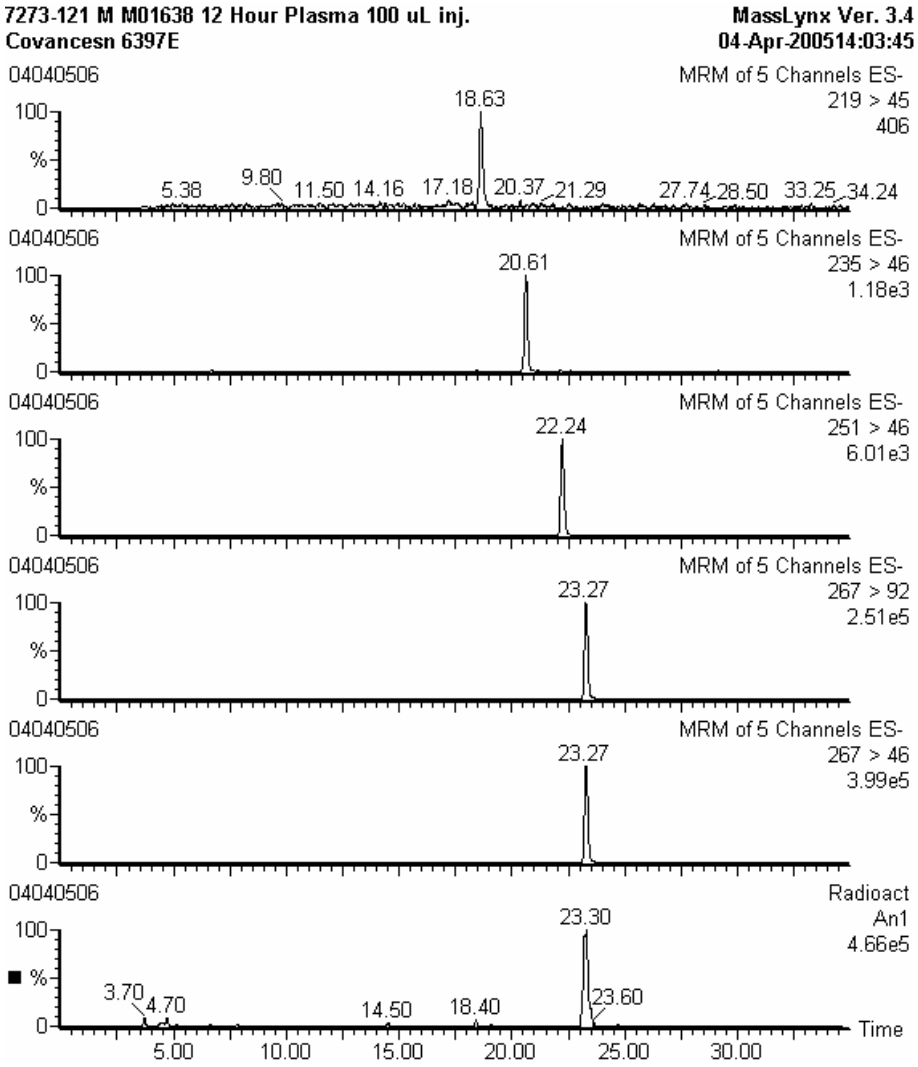
Component	Retention Time (minutes)	MRM Transition
TNX	18.58	219>45
DNX	20.56	235>46
MNX	22.20	251>46
RDX	23.24	267>92
RDX	23.24	267>46

Figure 4-18
MRM chromatograms and radiochromatogram from analysis of a 6 hour plasma sample from male Animal M01638 using LC/MS Method 2



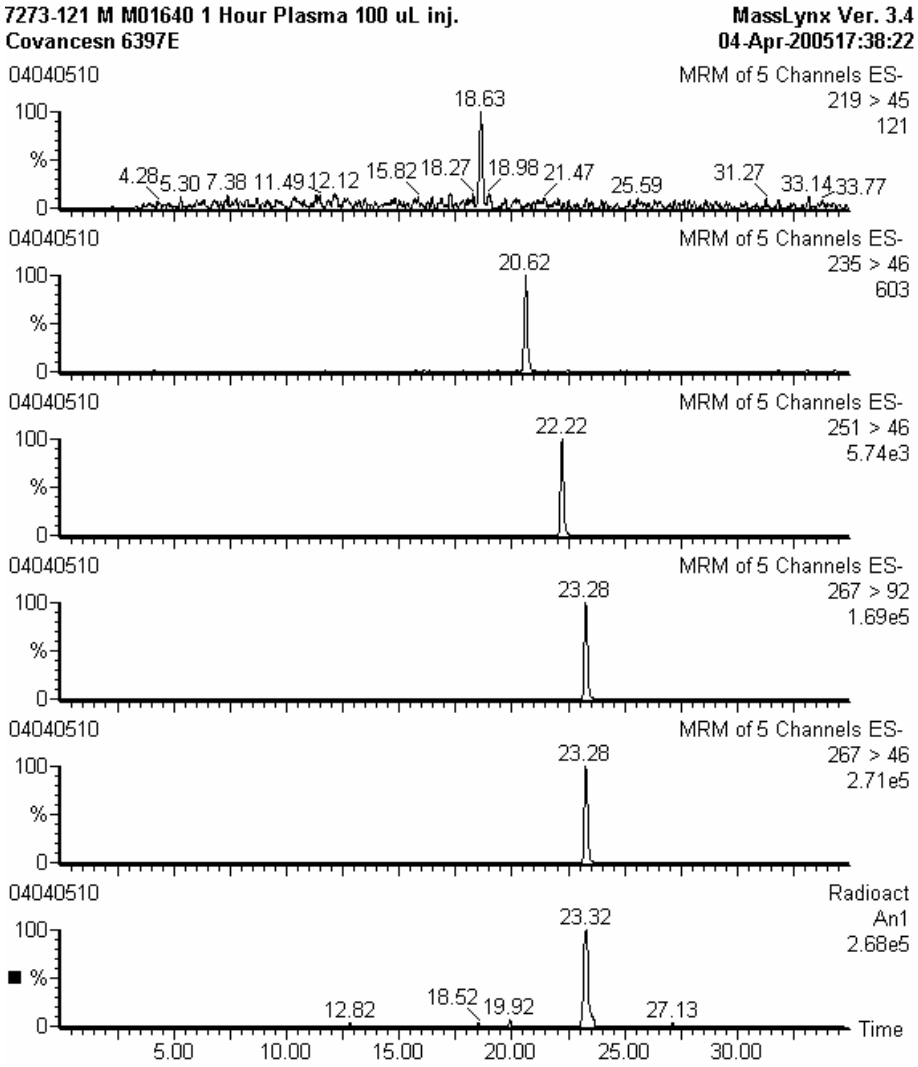
Component	Retention Time (minutes)	MRM Transition
TNX	18.62	219>45
DNX	20.61	235>46
MNX	22.22	251>46
RDX	23.28	267>92
RDX	23.28	267>46

Figure 4-19
MRM chromatograms and radiochromatogram from analysis of a 12 hour plasma sample from male Animal M01638 using LC/MS Method 2



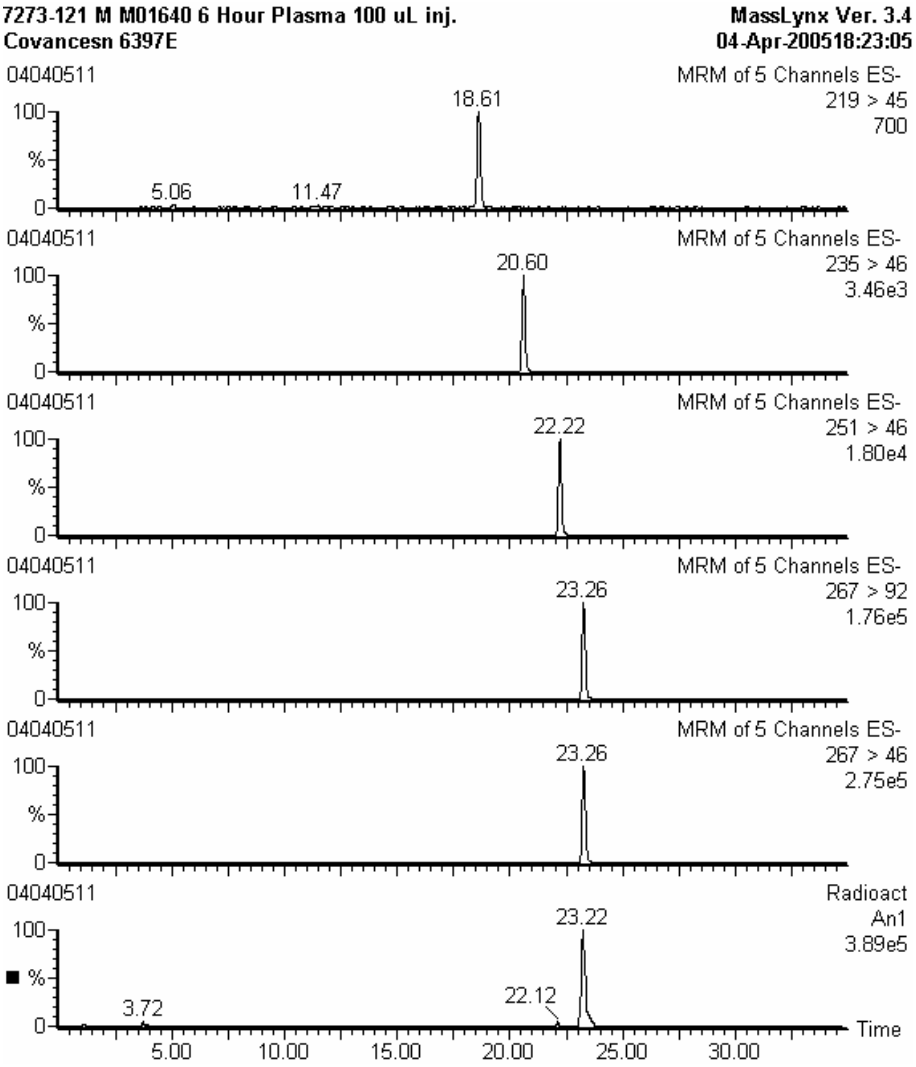
Component	Retention Time (minutes)	MRM Transition
TNX	18.63	219>45
DNX	20.61	235>46
MNX	22.24	251>46
RDX	23.27	267>92
RDX	23.27	267>46

Figure 4-20
MRM chromatograms and radiochromatogram from analysis of a 1 hour plasma sample from male Animal M01640 using LC/MS Method 2



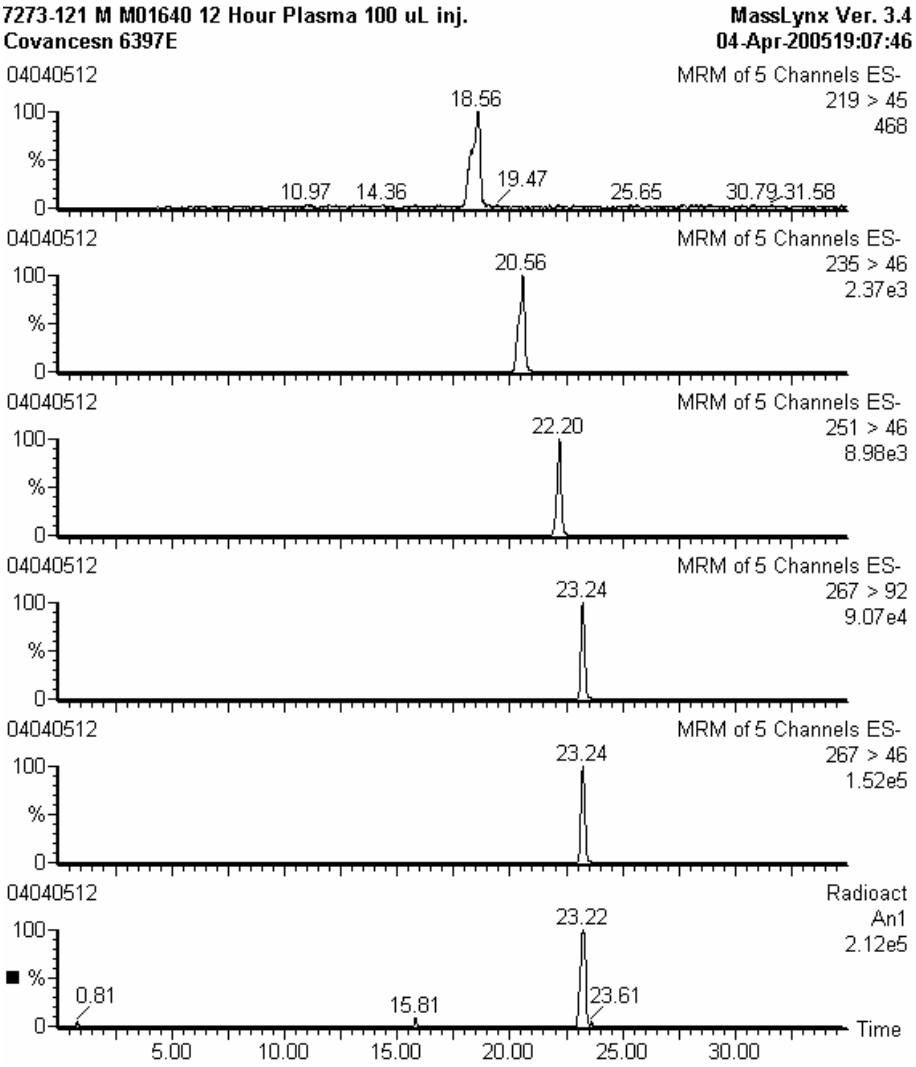
Component	Retention Time (minutes)	MRM Transition
TNX	18.63	219>45
DNX	20.62	235>46
MNX	22.22	251>46
RDX	23.28	267>92
RDX	23.28	267>46

Figure 4-21
MRM chromatograms and radiochromatogram from analysis of a 6 hour plasma sample from male Animal M01640 using LC/MS Method 2



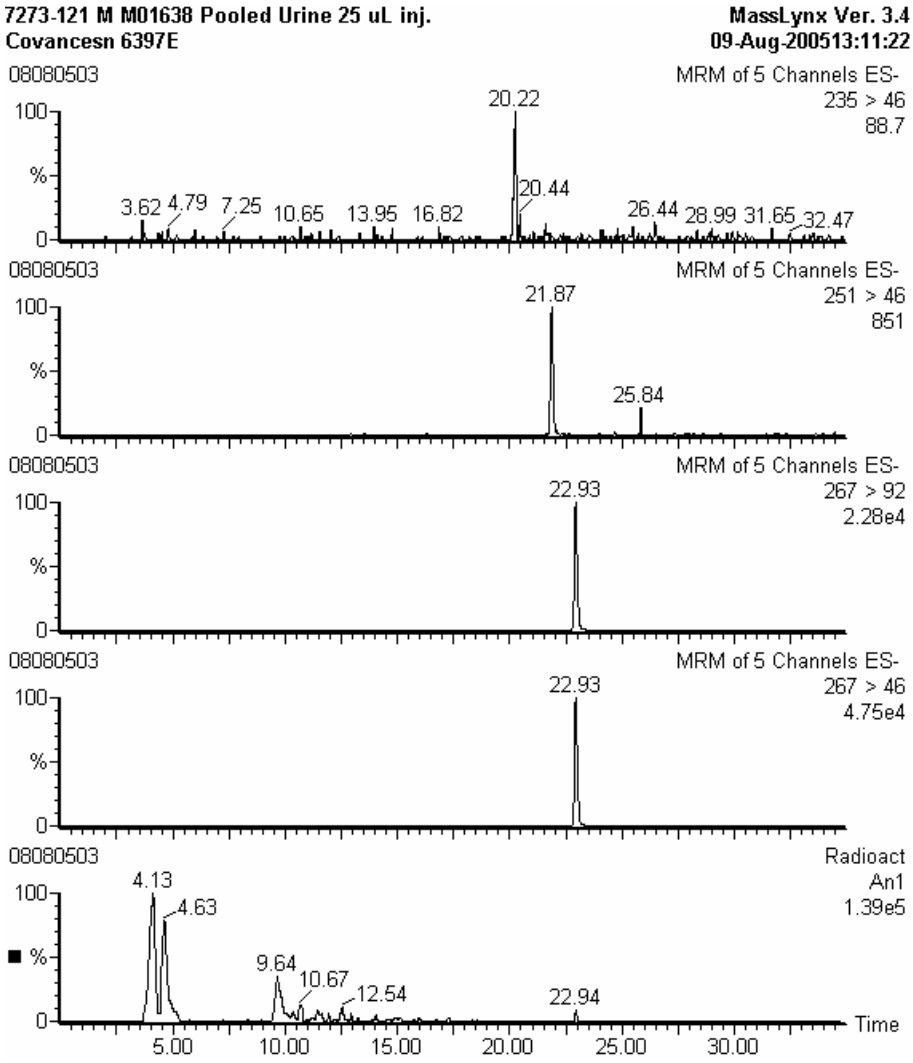
Component	Retention Time (minutes)	MRM Transition
TNX	18.61	219>45
DNX	20.60	235>46
MNX	22.22	251>46
RDX	23.26	267>92
RDX	23.26	267>46

Figure 4-22
MRM chromatograms and radiochromatogram from analysis of a 12 hour plasma sample from male Animal M01640 using LC/MS Method 2



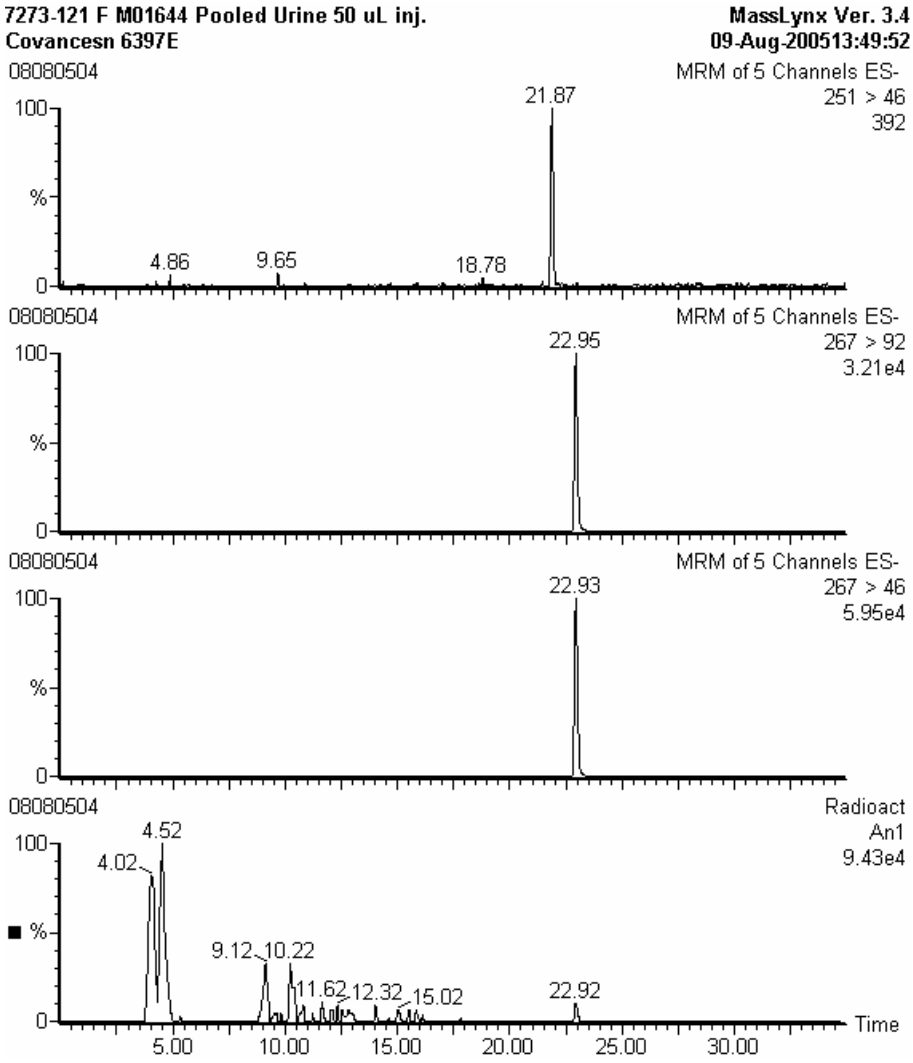
Component	Retention Time (minutes)	MRM Transition
TNX	18.56	219>45
DNX	20.56	235>46
MNX	22.20	251>46
RDX	23.24	267>92
RDX	23.24	267>46

Figure 4-23
MRM chromatograms and radiochromatogram from analysis of a pooled urine sample from male Animal M01638 using LC/MS Method 2



Component	Retention Time (minutes)	MRM Transition
DNX	20.22	235>46
MNX	21.87	251>46
RDX	22.93	267>92
RDX	22.93	267>46

Figure 4-24
MRM chromatograms and radiochromatogram from analysis of a pooled urine sample from female Animal M01644 using LC/MS Method 2



Component	Retention Time (minutes)	MRM Transition
MNX	21.87	251>46
RDX	22.95	267>92
RDX	22.93	267>46

Figure 4-25
Product ion (m/z 235) mass spectrum of DNX from analysis of a 12 hour plasma sample from male Animal M01638 using LC/MS Method 2

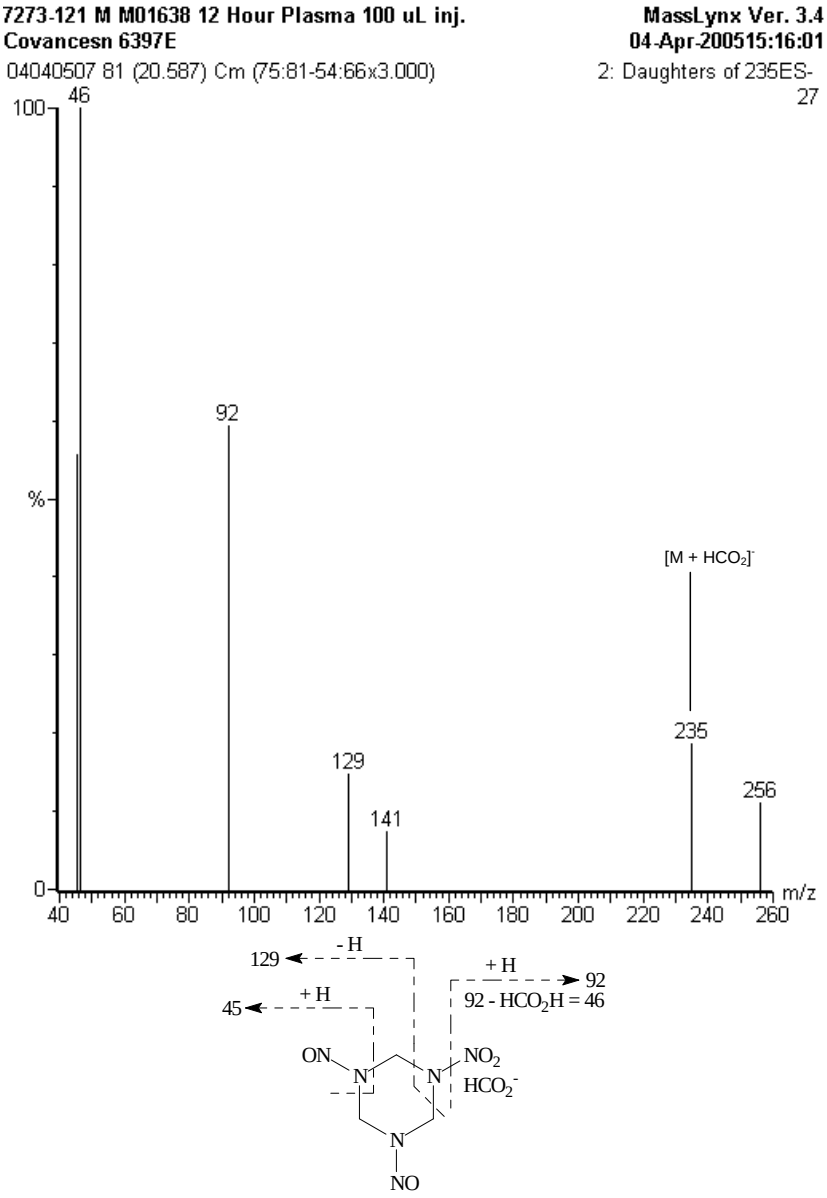


Figure 4-26
Product ion (m/z 251) mass spectrum of MNX from analysis of a 12 hour plasma sample from male Animal M01638 using LC/MS Method 2

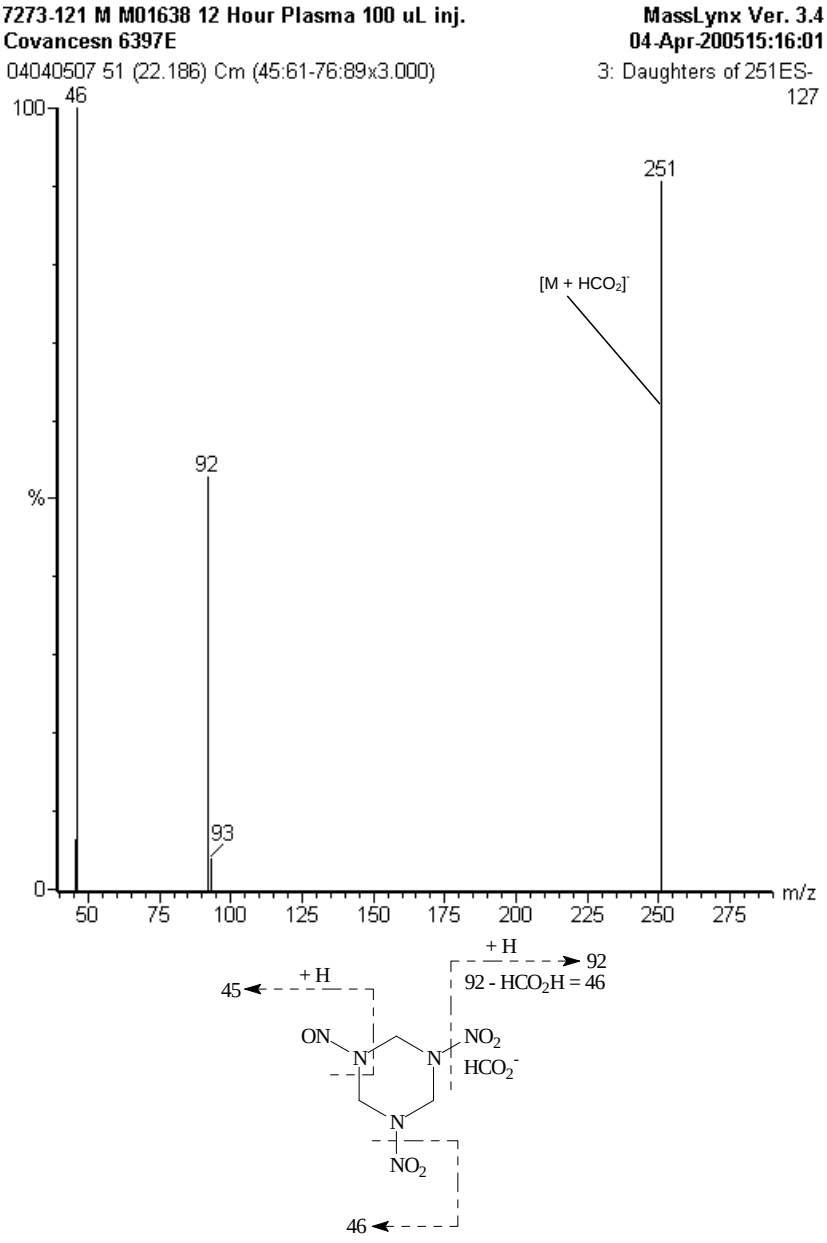
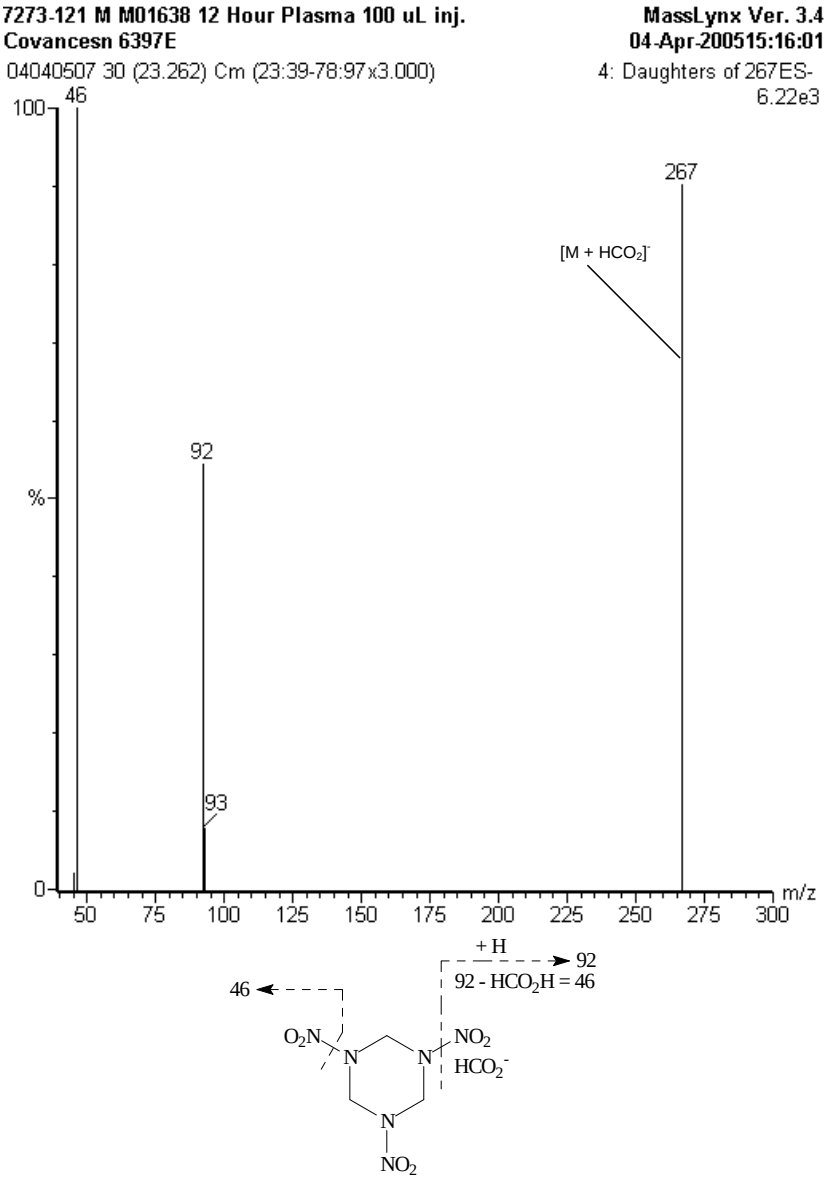


Figure 4-27
Product ion (m/z 267) mass spectrum of RDX from analysis of a 12 hour plasma sample from male Animal M01638 using LC/MS Method 2



Appendix 5
LC/MS Conditions for Quantitative Analysis of RDX in Brain and Liver

LC/MS/MS Analytical Method

Method Summary

Analyte:	RDX
Matrix:	Pig brain and liver
Extraction:	Protein precipitation
LC/MS/MS Instrumentation:	Sciex API-3000
Ionization Mode:	Electrospray negative

Stock Solution Preparation

Solution ID	Stock Concentration	Solvent
RDX	1000 µg/mL	ACN

Preparation of Intermediate Standard and Internal Standard Working Solution

Working Solution ID	Source Solution ID	Source Solution Concentration (µg/mL)	Source Solution Volume (mL)	Final Total Volume (mL)	Final Solution Concentration (ng/mL)	Solvent
Intermediate 1	RDX Stock	1000	0.192	3.00	64000	50:50 H_20:MeOH

H_2O Water.
MeOH Methanol.

Preparation of Calibration Standards

Working Solution ID	Source Solution ID	Source Solution Concentration (ng/mL)	Source Solution Volume (µL)	Final Total Volume (mL)	Final Solution Concentration (ng/mL)	Matrix
STD-1	Intermediate 1	64000	390.6	2.00	12500	MeOH:H_20 (50:50)
STD-2	Intermediate 1	64000	195.3	2.00	6250	MeOH:H_20 (50:50)
STD-3	Intermediate 1	64000	117.2	2.00	3750	MeOH:H_20 (50:50)
STD-4	Intermediate 1	64000	39.1	2.00	1250	MeOH:H_20 (50:50)
STD-5	STD-1	12500	80.0	2.00	500	MeOH:H_20 (50:50)
STD-6	STD-2	6250	40.0	2.00	125	MeOH:H_20 (50:50)
STD-7	STD-4	1250	40.0	2.00	25.0	MeOH:H_20 (50:50)
STD-8	STD-5	500	20.0	2.00	5.00	MeOH:H_20 (50:50)

H_2O Water.
MeOH Methanol.

All stock standard, intermediate and working solutions were stored at approximately 2-8°C.

Extraction Procedure

1	Include a reagent blank, and a blank matrix (double blank) with each calibration curve.
2	Spike 10 µL of appropriate calibration standard solution to 50µL of homogenized blank pig brain or pig liver. Aliquot 50 µL of homogenized study sample to 96 well plate, then add 10µL of 50:50 MeOH:H20.
3	To all samples, standards and blanks, add 200 µL of acetonitrile.
4	Vortex-mix all tubes for 30 seconds.
5	Centrifuge at 3000 rpm for 5 minutes.
6	Transfer 50 µL of the supernatant and mix with 150 µL of RO water in a 96-well elution plate.
7	Cover the plate and vortex-mix. Inject onto the LC/MS/MS system for analysis.

Chromatographic Conditions

Column:	Zorbax XDB-C18, 150 x 2.1 mm, 5µM particle size
Mobile phase A:	0.02% HOAC in water.
Mobile phase B:	0.02% HOAC in MeOH.
Flow Rate:	0.500 mL/min
Injection volume:	20 µL
Column temperature:	25°C

Gradient:

Time	%B	Valve switch
0.00	20	Waste
0.50	20	Waste
3.00	90	MS
4.00	90	MS
4.01	20	Waste
4.75		Stop

Run time: 4.00 minutes.

Mass Spectrometric Conditions (Sciex)

Instrument:	API 3000
Ionization mode:	TurboIonspray
Polarity:	Negative
Scan function:	Multiple Reaction Monitoring (MRM)
Parameters:	RDX
Precursor ion:	267.1
Product ion:	46.0
Dwell time (ms):	100
Declustering potential (V):	-15
Focusing potential (V):	-90
Collision energy (eV):	-28
Collision cell exit potential (V):	-8
Ionspray voltage (V):	-1500
Turbo gas temperature (°C):	450
Nebulizer gas:	8
Curtain gas:	8
Collision gas:	8
Resolution:	Unit
Software:	Analyst 1.4
Regression (weighting):	$1/x^{2,}$, Linear